Status: Path 1 of [Dialog Information Services via Modem]

Status: Initializing TCP/IP using (UseTelnetProto 1 ServiceID pto-dialog)
Trying 31060000009999...Open

DIALOG INFORMATION SERVICES
PLEASE LOGON:
 ******** HHHHHHHH SSSSSSSS?
Status: Signing onto Dialog
 ****
ENTER PASSWORD:
 ******** HHHHHHHH SSSSSSSS? ********
Welcome to DIALOG
Status: Connected

Dialog level 02.12.60D

Last logoff: 13apr03 15:06:32
Logon file001 14apr03 16:04:35
             *** ANNOUNCEMENT ***
                    ***
--File 515 D&B Dun's Electronic Business Directory is now online
completely updated and redesigned. For details, see HELP NEWS 515.
                    ***
--File 990 - NewsRoom now contains October 2002 to present records.
File 993 - NewsRoom archive contains 2002 records from January 2002-
September 2002. To search all 2002 records, BEGIN 990,993 or B NEWS2002
                    ***
--Alerts have been enhanced to allow a single Alert profile to be
stored and run against multiple files. Duplicate removal is available
across files and for up to 12 months. The Alert may be run according
to the file's update frequency or according to a custom
calendar-based schedule. There are no additional prices for these
enhanced features. See HELP ALERT for more information.
                    ***
--U.S. Patents Fulltext (File 654) has been redesigned with
new search and display features. See HELP NEWS 654 for
information.
                    ***
--Connect Time joins DialUnits as pricing options on Dialog.
See HELP CONNECT for information.
                    ***
--CLAIMS/US Patents (Files 340,341, 942) have been enhanced
with both application and grant publication level in a
single record. See HELP NEWS 340 for information.
                    ***
--SourceOne patents are now delivered to your email inbox
as PDF replacing TIFF delivery. See HELP SOURCE1 for more
information.
                    ***
--Important news for public and academic
libraries. See HELP LIBRARY for more information.
                    ***
--Important Notice to Freelance Authors--
See HELP FREELANCE for more information
                    ***
For information about the access to file 43 please see Help News43.
***
NEW FILES RELEASED
***Dialog NewsRoom - Current 3-4 months (File 990)
***Dialog NewsRoom - 2002 Archive (File 993)
***Dialog NewsRoom - 2001 Archive (File 994)
***Dialog NewsRoom - 2000 Archive (File 995)
***TRADEMARKSCAN-Finland (File 679)

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***TRADEMARKSCAN-Norway   (File 678)
***TRADEMARKSCAN-Sweden   (File 675)
                    ***
UPDATING RESUMED
***Delphes European Business (File 481)
                    ***
RELOADED
***D&B Dun's Electronic Business Directory (File 515)
***U.S. Patents Fulltext 1976-current (File 654)
***Population Demographics (File 581)
***Kompass Western Europe (File 590)
***D&B - Dun's Market Identifiers   (File 516)

REMOVED
***Chicago Tribune (File 632)
***Fort Lauderdale Sun Sentinel (File 497)
***The Orlando Sentinel (File 705)
***Newport News Daily Press (File 747)
***U.S. Patents Fulltext 1980-1989 (File 653)
***TOXNET data is added to ToxFile (F156)

***New document supplier***
IMED has been changed to INFOTRIE (see HELP OINFOTRI)


    >>> Enter BEGIN HOMEBASE for Dialog Announcements <<<
    >>>    of new databases, price changes, etc.        <<<
                    ****
KWIC is set to 50.
HILIGHT set on as '*'
* * * *


File   1:ERIC  1966-2003/Mar 24
        (c) format only 2003 The Dialog Corporation

    Set  Items  Description
    ---  -----  -----------
Cost is in DialUnits
?b 155, 159, 5, 73
        14apr03 16:04:51 User259876 Session D487.1
            $0.36    0.103 DialUnits File1
     $0.36  Estimated cost File1
     $0.06  TELNET
     $0.42  Estimated cost this search
     $0.42  Estimated total session cost   0.103 DialUnits


SYSTEM:OS  - DIALOG OneSearch
  File 155:MEDLINE(R)  1966-2003/Apr W1
        (c) format only 2003 The Dialog Corp.
```
File 155: Medline has been reloaded and accession numbers have
changed. Please see HELP NEWS 155.
```
  File 159:Cancerlit  1975-2002/Oct
        (c) format only 2002 Dialog Corporation
```
File 159: Cancerlit ceases updating with immediate effect.
Please see HELP NEWS.
```
  File   5:Biosis Previews(R)  1969-2003/Apr W1
        (c) 2003 BIOSIS
```
File   5: Alert feature enhanced for multiple files, duplicates
removal, customized scheduling. See HELP ALERT.
```
  File  73:EMBASE  1974-2003/Apr W1
        (c) 2003 Elsevier Science B.V.
```
File 73: Alert feature enhanced for multiple files, duplicates
removal, customized scheduling. See HELP ALERT.
```
    Set  Items  Description
```

```
      ---    -----  -----------
?s (dendritic (w) cell?) (s) ((multiple (w) antigens) or (array (w) of (w) antigens))
Processing
Processing
Processing
          87270   DENDRITIC
         8632717  CELL?
         1074138  MULTIPLE
          710382  ANTIGENS
             639  MULTIPLE(W)ANTIGENS
           75176  ARRAY
               0  OF
          710382  ANTIGENS
               0  ARRAY(W)OF(W)ANTIGENS
      S1       5  (DENDRITIC (W) CELL?) (S) ((MULTIPLE (W) ANTIGENS) OR
                  (ARRAY (W) OF (W) ANTIGENS))
?rd
...completed examining records
      S2       3  RD (unique items)
?t s2/3,k/all
```

2/3,K/1   (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.

09280394   21017579   PMID: 11143904
  Oral immunomodulation therapy in rheumatoid arthritis.
  Meyer O
  Service de rhumatologie, h pital Bichat, Paris, France.
  Joint, bone, spine - revue du rhumatisme (France)   2000, 67   (5)
p384-92, ISSN 1297-319X   Journal Code: 100938016
  Document type: Journal Article; Review; Review, Tutorial
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed

  ... present them to T cells. Intact antigens can penetrate through
specialized Peyer's patch enterocytes called 'M cells'; they are then
degraded and presented by *dendritic* *cells* to Peyer's patch T cells. The
influx of *multiple* *antigens* through the gastrointestinal mucosa usually
results in tolerance. High-dose tolerance is due to T cell deletion or
anergy, whereas low-dose tolerance involves activation...

2/3,K/2   (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.

08766970   20048304   PMID: 10581603
  Vaccine therapy for patients with melanoma.
  Haigh P I; Difronzo L A; Gammon G; Morton D L
  Sonya Valley Ghidossi Vaccine Laboratory, John Wayne Cancer Institute,
Saint John's Health Center, Santa Monica, California, USA.
  Oncology (Williston Park, N.Y.) (UNITED STATES)   Nov 1999, 13 (11)
p1561-74; discussion 1574 passim, ISSN 0890-9091   Journal Code: 8712059
  Document type: Journal Article; Review; Review, Academic
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed

  ...of the lack of effective conventional modalities. The most extensively
studied melanoma vaccines in clinical trials are whole-cell preparations or
cell lysates that contain *multiple* *antigens* capable of stimulating an
immune response. Unfortunately, in the majority of studies, immune
responses to these vaccines have not translated into a survival advantage.
Advances...

Oncologic, Endocrine & Metabolic

T-cell-directed cancer vaccines: the melanoma model

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Significant advances in the understanding of the molecular basis for tumour/host interactions in humans have occurred in the last decade through studying patients with metastatic melanoma. This disease is characterised by its tendency to be modulated by immunologic factors. Furthermore, immunologic manipulation of the host with various systemic agents, in particular IL-2, frequently affects this natural phenomenon and can lead to complete rejection of cancer. By studying the cellular immunology occurring in patients undergoing immunotherapy, several tumour antigens (TA) and their epitopes recognised by human leukocyte antigen (HLA) class I-restricted cytotoxic T-lymphocytes (CTL) have been identified. Most of these TA are non-mutated molecules expressed by the majority of melanoma *in vivo* and most melanoma cell lines. In addition, unique minimal epitopic sequences play an immunodominant role in the context of specific HLA class I alleles. Since melanoma lesions from different patients often share expression of the same TA, and a minimal peptide sequence from a TA can cause immunologic changes in multiple patients, interest has grown in the development of TA-specific vaccines suitable for broad patient populations. Repeated *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) with TA-derived epitopes can induce a high frequency of TA-reactive T-cells in melanoma patients. The same epitopes can also enhance TA-specific T-cell reactivity *in vivo* when administered subcutaneously in combination with Incomplete Freund's Adjuvant (IFA). Epitope-based vaccinations, however, have not shown strong clinical efficacy unless combined with IL-2 administration. Attempts to increase the efficacy of these vaccines have combined specialised antigen-presenting cells or the administration of whole TA through DNA- or RNA-based vaccines with the intention of increasing antigen presentation and processing. Save for scattered reports, however, the success of these approaches has been limited and T-cell-directed vaccination against cancer remains at a paradoxical standstill whereby anticancer immunisation can be induced but it is not sufficient, in most cases, to induce tumour regression. Using melanoma as the standard model for immunotherapy, we will review various methods of T-cell-directed vaccination, the monitoring and analysis of the resulting immune response, and several clinical trials in which cancer vaccines have successfully induced immunisation.

Keywords: *immune monitoring, immunotherapy, melanoma, neoplasm, T-lymphocyte, vaccines*

1. Introduction

The immune response against pathogens utilises both a humoral and a cellular arm. While the former is directed toward extracellular pathogens, the latter is directed toward proteins produced by infectious agents replicating within permissive host cells. Intracellular proteins produced by infectious agents are enzymatically degraded into short peptides (9 - 11 amino acids in length) and presented on the surface of infected host cells in association with Major Histocompatibility Complex (MHC) class I molecules. This MHC complex is required for triggering T-cells through direct interaction with the T-cell receptor (TCR). Most tumour antigens identified so far are intracellular molecules. Thus, the cellular immune response is likely the prevalent immunologic defence against cancer [1] since antibodies cannot penetrate the intracellular compartment.

Using IL-2, stable human T-cell lines specifically recognising autologous tumour cells could be expanded *in vitro* from excised tumour specimens [2]. Due to the capacity of IL-2 in enhancing T-cell activation, proliferation and antitumour activity *in vitro*, IL-2 was administered to patients with advanced cancer [3], in particular metastatic melanoma and renal cancer for which no effective alternative therapy is available [4]. Administration of high-dose iv. IL-2 resulted in 7% complete and 10% partial tumour regressions in patients with metastatic melanoma [5]. IL-2 was then used to expand *in vitro* Lymphokine-Activated Killer (LAK) cells from peripheral blood monocytes (PBMC) [6] and tumour-infiltrating lymphocytes (TIL) from tumour specimens for adoptive cell transfer [7]. While LAK cells did not prove useful in randomised clinical trials [6], the adoptive transfer of TIL suggested an additional clinical benefit over of IL-2 alone inducing a 34% objective response rate [5].

Due to these promising results and the ease with which tumour-specific TIL could be obtained from melanoma patients, melanoma has served as the prototype human model for studying tumour immunology. Molecular characterisation of TIL led to identification of TA [2,7,8]. Kawakami *et al.* had shown that TIL could kill melanoma cell lines from different patients provided they expressed at least one matched MHC (called Human Leukocyte Antigen or HLA in humans) class I molecule [9]. Based on this HLA 'restriction,' TA were identified. HLA-restricted recognition of TA was formally proven by transduction of a specific HLA allele into melanoma cells lacking the 'restricting element' that was associated with recognition of the TA by a particular TIL [9]. After establishment of a cDNA library from the melanoma cell line recognised by the TIL, genes from the library were transfected into non-melanoma cells expressing the correct restriction element. The transfected target cells were then tested for recognition by the same TIL and the cDNA clones that caused recognition were isolated and sequenced for identification of the TA [5,10].

2. Tumour antigens

Two major categories of TA were identified. The first category includes tumour differentiation antigens (TDA), such as MART-1/MelanA, gp100/Pmel17, tyrosinase, TRP-1 and TRP-2. TDA are expressed by both melanoma cells and normal melanocytes [11-14] but not other malignancies and normal tissues [15]. The second category involves tumour specific antigens (TSA), which are found in normal gametic cells in the testes and in cancers in addition to melanoma but are not expressed by normal melanocytes. TSA include the MAGE, BAGE and GAGE families and NY-ESO-1 [16]. NY-ESO-1 was found in approximately 30% of melanomas and breast tumours and occasionally identified in malignancies of the lung, liver, thyroid, ovary and prostate [16]. Therefore, ESO-1 is a promising candidate tumour antigen and is currently the focus of numerous clinical trials.

Due to the prevalence of HLA-A*0201 in the melanoma population (~ 50%), a large number of TA were identified in association with this allele [17,18]. Among them, MART-1 has received particular attention because of its 'immunodominance' in the context of HLA-A*0201 [19]. It was originally noted that 90% of TIL expanded from HLA-A*0201 patients recognise the MART-1:27-35 epitopic sequence [20]. This work was based on the analysis of archival TIL strains maintained in culture for several passages. More recent work by Kawakami *et al.* extended the analysis of specificity to a broader population of short-term HLA-A*0201-associated TIL cultures and suggested that the rate of MART-1 recognition is not as high as originally believed [21]. We have also noted, by directed enumeration of MART-1-specific TIL with epitope/HLA tetrameric complexes, that the frequency of MART-1-specific TIL is also not as high as predicted by those original studies [22]. Another commonly recognised antigen in the context of

HLA-A*0201 is gp100. Analysis of 217 fresh metastatic melanoma specimens demonstrated that, although the expression of MART-1 and gp100 *in vivo* can be quite heterogeneous, the majority of melanoma metastases express these TA [13]. Due to their frequency of expression, the main focus of vaccination efforts has been directed towards these two TA.

2.1 Peptide-based vaccines

Extensive analyses have shown that the MART-1:27-35 epitope is consistently recognised by MART-1-specific, tumour-recognising HLA-A*0201 restricted T-cells [23]. More than one HLA-A*0201 restricted peptide could be identified for the larger gp100 [24]. However, gp100 epitopes were not as efficient as MART-1:27-35 for *in vitro* induction of CTL. Thus, to enhance the immunogenicity of gp100 epitopes, single amino acid substitutions were made to increase the binding affinity to HLA-A*0201. A modified peptide was subsequently identified, gp100:209-217(210M) (g209-2M), with sequence IMDQVPFSV, modified from its natural sequence: ITDQVPFSV), with increased immunogenic potential *in vitro* and *in vivo* [25,26]. Since HLA-A*0201 is the predominant allele in the melanoma population [17], peptide-based vaccinations restricted to HLA-A*0201 patients were initiated by sc. administration of MART-1:27-35 emulsified in IFA. Comparison of reactivity of PBMC obtained before and after vaccination demonstrated strong enhancement of immune-competency toward MART-1 [27]. In a second trial, melanoma patients were treated with g209-2M alone or in combination with high dose iv. IL-2. Successful immunisation could be documented in patients immunised with g209-2M; however, no objective clinical responses were observed unless IL-2 treatment was added [28]. CTL has also been induced in patients receiving tyrosinase vaccination; in one study regression of a metastasis was noted in a patient with persistence of tyrosinase expression [29].

2.2 Dendritic cell-based vaccines

Preclinical studies suggest that the administration of peptide alone for the treatment of cancer is not as efficient as when an appropriate adjuvant was added [30]. Among adjuvants, dendritic cells (DC) play a critical role because they are highly specialised antigen-presenting cells (APC) with unique immunostimulatory properties. DC can induce primary cellular immune responses [31]. Activated DC can migrate from areas of antigen capture in the peripheral tissues to areas infiltrated with naive T-cells, such as of lymphoid organs. DC have been shown also in preclinical models to play a role in class I-restricted antitumour sensitisation *in vivo* [32-38] and as an addition to immunisation with peptide alone [33,36,37]. These preclinical successes have led to peptide-pulsed DC-based clinical trials with varying results [39,40].

DC cultured in IL-4 and GM-CSF could sensitise *in vitro* PBMC from melanoma patients against HLA-A*0201-restricted epitopes, including MART-1:27-35 and g209-2M, by a single exposure of the responder cells to the relevant TA [41]. These findings stimulated a Phase I clinical trial at the National Cancer Institute (NCI) in which patients with metastatic melanoma were immunised with DC pulsed with MART-1 and gp100 epitopes. Previously reported clinical studies had generally used minimal numbers of DC compared with the potential yield of DC from a standard leukapheresis and had applied different routes of administration [39,40]. In the NCI study, up to 2×10^8 DC were safely administered intravenously. However, with the exception of one patient who experienced a temporary partial regression of sc. and pulmonary metastases, no clinical or immunological benefits were noted [13]. Nestle *et al.* reported high rates of tumour regression in response to the intra-lymphatic administration of a small number of DC prepared with methods similar to the those used in the NCI study and pulsed either with TA derived peptides or with tumour preparations [39]. Thurner *et al.* vaccinated eleven patients with MAGE-3A1 peptide-pulsed DC and was able to expand CTL in eight patients and reported clinical regression in six patients [42]. The different routes of DC administration, maturation status of DC and the addition of helper antigens including KLH and TT may be responsible for the significant differences in clinical outcomes of these DC trials.

2.3 Whole antigen vaccines

Usage of TA-derived peptides requires knowledge of the amino acid sequence of the epitope specific for each HLA allomorph [43]. Various strategies have been reported to obviate this problem. The use of acid-eluted peptides derived from autologous tumour has been proposed [35]. Other strategies have taken advantage of the ability of APC to incorporate exogenous particles or messenger-RNA (mRNA) and present them to T-cells [44,45]. Bhardwaj *et al.* [46] demonstrated that DC can be infected by viruses and

be permissive to the expression of viral products. We infected DC with viral constructs encoding TA to stimulate autologous human T-cells and observed that DC are permissive to poxvirus-driven expression of TA [41,47]. Virally-induced TA were naturally processed by DC and presented as relevant epitopes [47] which could efficiently induce TA-specific T-cells [41].

With virally-infected DC we extensively analysed the stringency of HLA allele/epitope requirements for immunogenicity of a particular antigen/HLA combination [48]. Those studies demonstrated that virally-induced TA-specific CTL by autologous DC is restricted to a unique allele/ligand combination and is excluded by minimal changes in HLA structure. Thus, the use of whole TA, including epitopes associated with multiple restriction elements, may not be as useful as theoretically predicted [49,50]. Adenoviral vectors have been tested in Phase I clinical trials. One of 16 patients receiving adeno-MART-1 experienced a complete clinical response with disappearance of all evaluable metastases. Other objective clinical responses occurred in patients receiving IL-2 simultaneously and thus could not be attributable specifically to the viral-TA vaccine. In addition, no consistent evidence of immunisation to MART-1 or gp100 could be demonstrated in contrast to the results described for peptide vaccines.It is possible that neutralising antibodies generated by the exposure to the virus eliminated the vector before it could generate antigen for immunisation [51].

2.4 DC/tumour hybrids

Some have advocated the construction of TA-presenting cell hybrids with the intent of combining the potency of DC as professional antigen-presenting cells with the unlimited antigenic potential of cancer cells. This approach has the obvious advantage of not requiring the molecular identification of all potential antigens expressed by each patient's cancer cells. Promising results were reported recently by hybridising DC with renal cancer cells [52]. Similarly, others attempted to hybridise autologous cancer cells to heterologous melanoma cells from established cell lines [53]. This approach has several advantages. It allows the expansion of autologous tumour cells in those cases when little starting material is available since the efficiency of the hybridisation is very high. In addition, the hybrids carry foreign HLA alleles that can induce a strong

immunologic reaction at the site of inoculation that may increase the potency of the vaccine.

3.Monitoring immune response and heterogeneity

3.1 Monitoring of anticancer vaccines

The ultimate goal of a vaccine is to promote cancer rejection. However, the biological goal is to increase the antitumour immune competence of the host. Antigen-specific vaccinations, although clinically disappointing, have given a unique opportunity to test localisation and activation status in the target organ of vaccine-specific systemic T-cell responses. At the same time, accurate documentation of the expression of molecules targeted by the vaccination can be performed. Thus, analysis of these molecular treatments allows the opportunity to directly measure immune responses that may in turn provide further insight into the biology of tumour rejection and suggest more effective methods of therapy.

To fully assess the effects of a cancer vaccine, laboratory studies are focused on monitoring the parameters of vaccine administration, biological properties and local or systemic effects. The assessment of patient and tumour-related factors suspected to influence antitumour immune response should be considered. It is essential to demonstrate that the vaccine induces an immune response that recognises the antigens as naturally processed and presented by tumour cells. Furthermore, assessment of the magnitude and quality of the immune response is likely to be important in determining the potential efficacy of the vaccine. For example, the number of TA-specific cells [54], their avidity for the antigen [55], the level and variety of cytokines they produce [56] and their tumour localisation may be critical for mediating antitumour effects [57].

Theoretically, the tumour is the most relevant tissue for monitoring immune responses, since lymphocytes or antibodies must reach the tumour in order to mediate antitumour effects. Examination of the tumour can also confirm that the relevant antigen and the associated HLA molecules are expressed. Post-vaccination sampling of tumour specimens can provide important information on immune selection. Even when this is feasible, an unsolved question is the timing of tissue sampling during vaccine administration. However, in most circumstances, it is only

possible to monitor specific responses in peripheral blood. This information verifies that the vaccine could induce a systemic immune response.

3.2 Patient heterogeneity in immune responses

Induction of an immune response may depend on patient-specific factors including prior treatment with cytotoxic agents, type and stage of disease, performance status etc. Some investigators have reported generalised or specific defects in T-cell signalling in patients with advanced cancer [58]. Defects in the maturation of professional APC due to tumour overproduction of vascular endothelial growth factor have also been reported [59]. Moreover, there are no reliable tests to evaluate immune competence and the ability to respond to antigen exposure. Many investigators rely only on general markers of immune responsiveness, such as delayed type hypersensitivity, CD4+/CD8+ ratios or lymphocyte responsiveness to mitogens, viral antigens or alloantigens. The relevance of these studies to assess antigen-specific immune competence, however, remains undetermined.

High resolution HLA typing is an important aspect of patient selection due to the HLA restriction of some vaccines [60]. Patients selected for treatment with a particular HLA-restricted peptide antigen must be shown to express that HLA molecule. Such a requirement may not be necessary when the immunising preparation is a whole protein or is derived from whole tumour cells. However, because of the difficulty in measuring and characterising immune responses to proteins or whole tumour cells, monitoring is often directed to specific HLA-restricted epitopic determinants of the proteins contained in the vaccine [51,61].

3.3 Tumour heterogeneity and immune response

Clinical data suggest the possibility of dissociation between immune responses detected in peripheral blood *versus* tumour [62]. Thus, assessment of tumour/host interactions at the tumour site can guide selection of appropriate patients for immunologic treatment and provide more accurate information about the biological basis for treatment failures and successes. Unfortunately, obtaining sufficient tumour tissue for these studies can be difficult, particularly when the tumours are not easily accessible. Furthermore, assays conducted on tumours are expensive and labour-intensive and interpretation of the results

is complicated by heterogeneity within and between tumour specimens.

Fine needle aspirates (FNA) of tumours, which can be performed with relative ease and minimal morbidity, can provide sufficient tissue for some correlative studies and give information on TA and HLA expression. In addition they can be used to establish both lymphocyte cultures and tumour cell lines for *in vitro* assays [63]. A number of other tumour-related factors have been identified that may determine responsiveness to a vaccine, including tumour expression of immunosuppressive cytokines and apoptotic signals or lack of expression of adhesion molecules on tumour vasculature that allow penetration by T-cells [64]. Due to the many factors that appear to impact on the antitumour immune response [65], newer technologies, such as gene chip arrays, which can measure expression of many genes simultaneously within a tumour tissue sample, may be necessary to fully characterise the tumour phenotype prior to immunisation [66].

3.4 Monitoring immune responsiveness at global transcript level

The identification of TA has provided a tool suitable for dissecting the molecular immunology of tumour/host interactions [67,68] by focusing on one single clearly defined target molecule at a time. However, as the resolution of our insight increases, new questions emerge regarding the natural history of immune-mediated adjustments of tumour phenotypes and, conversely, tumour-induced adaptation of the host immune competence [65,69]. As previously discussed, clinical studies have raised new questions based on the paradoxical observation that in several instances the induction of tumour-reactive circulating T-cells by the vaccine does not correlate with clinical effectiveness [27]. This discrepancy suggests that a clearly defined therapy against specific biological targets, which should lead to cancer rejection, is insufficient. Also, it represents a highly relevant example of the complexity of human cancer and the biological process underlying it. An extensive number of events downstream of the generation of tumour-reactive lymphocytes might explain the unpredictable behaviour of supposedly immune-responsive human cancers [65].

Understanding of the biological phenomena associated with tumour rejection in response to immune manipulation therefore will depend on new

technologies that allow for the global evaluation of thousands of gene interactions at one time [70]. We have paid particular attention to microarray technology, which allows comparative measurement of the expression of thousands of genes in relation to a biological process [71]. Among the various methods introduced, the utilisation of partial cDNA sequences from genes with known function or from expressed sequence tags (ESTs) from uncharacterised genes has been particularly successful [72]. These cDNA are spotted on a solid surface similar to a standard pathology slide. Total RNA or poly(A)-RNA from a tissue or cell line is then converted into cDNA, labelled with a reporter fluorescent molecule and then hybridised to the slide containing the arrayed genes. mRNA from a reference tissue or cell line is co-hybridised to the same slide after labelling with another fluorochrome. The addition of a consistent reference system allows correction for technical variability among hybridisations. Advances in computing power [73] and statistical tools [74] allow efficient interpretation of the extensive data derived by these analyses and allow discrimination between true expression patterns and random artefacts.

Advanced statistics can also characterise patterns of gene expression relevant to various biological processes [71,75,76]. Global patterns of gene expression could be obtained of neoplastic processes [77] and modulation of T-cell function [78]. Furthermore, 'portraits' of tumour specimens could be derived by the study of surgical specimens [79-81]. These efforts have identified molecular subsets of cancer based on mathematical analyses of their extended gene expression profile [77,79,80,82-84]. With the hope of identifying subcategories of disease with homogeneous behaviour, natural or treatment-induced, Clark et al. identified several genes associated with metastatic potential in a murine model of human melanoma [85]. Bittner et al. described two biologically distinct molecular profiles of cutaneous melanoma with divergent metastatic potential in vitro; but due to limitations in the study design, no clinical correlates could be identified [80]. More recently, Perou et al. separated primary breast carcinomas into two groups with molecular portraits suggesting diversity in expression of markers potentially · correlating with survival [81]. However, a direct association between the molecular profile of a solid tumour and its biological behaviour has not been as yet described. Our work suggests that melanoma metastases diverge biologically in 20 - 30% of patients

[13]. In addition, although Perou et al. [81] noted that primary breast tumours biopsied pre- and post-treatment do not change significantly during a short observation period, we and others have noted in metastatic melanoma that specific biological markers may vary significantly [62,86].

The difficulty of correlating laboratory findings with clinical outcome is a significant obstacle to the assessment of the role of immune escape and/or tolerance in cancer progression. Tumour/host interactions are generally studied in excised surgical specimens. These specimens, however, are not optimal for functional studies addressing the status of T-cell activation in situ. Freshly isolated tumour cells or lymphocytes are sub-optimal for accurate functional studies due to their extensive contamination by various cell types and the altered conditions of cells recently subjected to enzymatic or mechanical treatment. Expansion of TIL/tumour cell pairs provides elegant models for the characterisation in vitro of CTL/tumour interaction but these studies are only indirectly representative of in vivo conditions [87]. In addition, analysis of reagents obtained from excised specimens yields static information about a disease characterised by extreme genetic instability [88]. By excising the tumour mass, its natural or therapy-induced behaviour cannot be followed prospectively and an assumption is made perhaps erroneously that the excised lesion is representative of other lesions left in vivo. This limitation could be overcome by serial analyses of identical tumour samples through FNA, which provide the opportunity to evaluate dynamically the expression of relevant markers [12,62]. Due to the limited amount of material obtainable, however, FNA suffers from its own limitations. FNA could be combined with other techniques allowing analysis of limited materials. Distinct populations of cells could be sorted by micro-dissection [89,90] or epitope/HLA tetramers [91,92] and their status of activation could be tested using accurate and sensitive methodologies, such as Taqman-based real time RT-PCR [93] or intracellular FACS analysis [94]. This theoretically allows evaluation of the status of activation of CTL in vivo. Collection of cDNA libraries from FNA of metastases could profile patterns of expression of thousands of genes in a single experiment [66]. This information, combined with knowledge of the natural history of the lesion left in situ, might yield clinical material for the correlation of laboratory findings with clinical outcome and identification of the algorithm necessary for tumour

regression. Recent work in our laboratory has shown that the use of FNA-derived material may yield useful information about the kinetic of the interaction between the host immune system and cancer [63,95].

Since, of the increasing number of biological variables that could play a role in modulating tumour immune responsiveness, we believe that a global approach to the problem using microarray technology should be adopted. Although the *ex vivo* analysis of tissue samples using cDNA microarray technology is somewhat limited by the amount of RNA necessary for conventional cDNA microarrays (50 - 200 µg of total RNA or 2 - 5 µg poly(A)-RNA) corresponding to 106 - 107 cells sufficient for conventional microarray analysis. This number of cells is above the yield from FNA or micro-dissection specimens. To broaden the use of cDNA microarrays to experimental conditions in which source material is the limiting factor, we recently described a procedure that allows 105-fold enrichment of source RNA by combining antisense RNA (aRNA) amplification with template-switching effect to generate full-length double-stranded cDNA [96]. In this method, the amplified cDNA collection still retains the relative proportions of the original mRNA population and then is used as a target in cDNA microarrays. This allows analysis of global gene expression profiles in samples from FNA throughout the natural history of a tumour and/or in response to treatment. In addition, this technique might be utilised on micro-dissection specimens in which a finite number of cells (< 1000) are obtainable.

4. Assays of bulk cultures of peripheral lymphocytes

Immunisation against various antigens in animal models and humans has been shown to produce expansion of TA-specific T-cell precursors in PBMC. Comparative analysis of pre- and post-vaccination PBMC cultures is used to demonstrate expansion of vaccine-specific T-cells. Detection of T-cell reactivity in bulk cultures often requires repeated exposure of the T-cell precursors *in vitro* to the TA relevant to the vaccine in combination with IL-2 or other cytokines. Although lymphocytes can produce several cytokines, assays for detection of antigen-specific cells in bulk cultures are often designed to measure IFN-γ release upon antigenic stimulation or cytotoxicity of TA-bearing targets. By using these detection methods, successful immunisation to the MART-1

peptide was first demonstrated [27]. Characterisation of CTL responses in bulk culture can be expanded to estimate the avidity of the CTL for the vaccine. Detection of CTL activity at low E:T ratios, or recognition of peptide-pulsed target cells at very low concentrations of peptide, suggests that CTL have high avidity for the antigen-HLA complex [97]. Regardless of the methodology, the ultimate purpose of immune monitoring is to develop laboratory surrogate markers of clinical response.

4.1 ELISPOT assays

The ELISPOT assay was developed to provide quantitative evaluation of antigen-specific T-cell frequency [98]. Plates are coated with antibody to a specific cytokine. Target cells pulsed with peptide, or target tumour cells, are placed onto the plate, upon which the lymphocytes are added. Plates are incubated for approximately 24 - 48 h. Cytokines released by lymphocytes specific to the target will be captured by the pre-coated antibody and specifically detected by colourimetric assay. The cytokine spotted colonies are then counted and the total number of spots in plates loaded with the relevant target minus the spots formed in the plates with a non-relevant target is used to estimate the frequency of antigen-specific T-cells.

The published experience using the ELISPOT to monitor T-cell responses to cancer antigens is still limited [99]. ELISPOT assays conducted by Pass *et al.* in patients immunised with peptides derived from gp100 demonstrate that an 8 - 12 day *in vitro* sensitisation (IVS) with peptide and IL-2 was necessary to detect reactivity above background. After the IVS, peptide-reactive CTL could be detected in most patients immunised with the gp100:209-217(210M) modified peptide or the native gp100:209-217 peptide and in approximately 20% of patients immunised with the gp100:280-288 (288V) peptide. The frequency of CTL after the 8 - 12 day of IVS was in the range of 0.1 - 1%. When tumour was used as the target in the ELISPOT, reactive CTL at a frequency of 0.1 - 1% were demonstrated in several patients immunised with the modified gp100 peptide.

4.2 Limiting dilution assays

The limiting dilution assay (LDA) was also designed to yield an estimate of T-cell precursor frequency in the circulation and therefore to be a relatively quantitative assay. Furthermore, as expansion of T-cells is integral to the conduct of this assay, it has the capacity for increased sensitivity compared with assays, such as

ELISPOT, intracellular cytokine and tetramer analysis. The number of 'negative' cultures per dilution of the starting lymphocyte population can be plotted and statistical methods can be employed to determine the frequency of the target antigen-specific lymphocytes in the undiluted lymphocyte population obtained from peripheral blood [100]. Each culture per dilution is set up by adding feeder cells (usually irradiated PBMC) and then adding the antigen of interest (a peptide in many cases) or a tumour target. A parallel series of control cultures are established with irrelevant targets. The lymphocytes are added at a predetermined E:T ratio along with IL-2. After allowing a sufficient period of time for the antigen-specific lymphocytes to proliferate, labelled target cells containing the antigen are added to the cultures to test for specificity of each colony.

The results of the LDA depend on several variables including the type of cellular response that will be assayed, the target cells, the frequency of the antigen-specific cells in the starting population, the ability of those cells to expand in culture and the number of cultures established per dilution as well as the number of dilutions that are examined. Overall, the sensitivity of LDA appears to be superior to other assays that use a functional end point for detection of antigen-specific lymphocytes, with a range of 1/30,000 - 1/100,000. Even under the most favourable circumstances, however, measurement of lymphocyte responses with the LDA is labour-intensive and not practical for routine monitoring of cancer vaccine trials.

4.3 Intracellular cytokine production

A more direct assay quantifies TA-specific T-cells on the basis of intracellular cytokine production. T-cell membrane permeability is increased by chemical means, which allows penetration and binding by antibodies specific to a cytokine, usually IFN-γ. Multicolour flow cytometry can then be used to quantify and separate subsets of activated T-cells among a bulk PBMC population. The technique has not been fully developed nor used extensively to date in monitoring of cancer vaccine trials and is limited in overall by the sensitivity of flow cytometry [101].

4.4 Tetramer assays

Another direct assay enumerates *ex vivo* T-cells containing TCR capable of binding to a relevant peptide-MHC complex. When a lymphocyte TCR binds to only a single peptide-HLA complex with relatively low affinity, it rapidly dissociates. However, when 2 - 4 HLA molecules are linked and the same peptide antigen is properly bound to the HLA molecules, the dissociation rate of is diminished substantially, allowing for identification and sorting of epitope-specific lymphocytes by FACS [91]. The sensitivity of peptide-HLA tetramers in detecting TA-specific CTL is greater than other direct functional assays, since the detection of T-cells is based primarily on the binding properties of the tetramer with the TCR. Indeed the only limit of detection is due to the sensitivity of the FACS instrument, which can reliably detect approximately 1/5000 - 1/10,000 cells (0.01 - 0.02%). The peptide-HLA tetramers provide reliable and direct measurements of TA-specific T-cells directly on peripheral blood or tissue. One major drawback to tetramer analysis, however, is the requirement for a defined epitope. Since melanoma is the only tumour type in which immunodominant putative TA are known, this type of analysis has exciting yet narrow applicability.

The published experience with tetramers to monitor CTL responses to cancer antigens remains limited [92,102]. Romero *et al.* demonstrated higher frequencies of MART-1/Melan-A CTL in CD8+ lymphocytes obtained from melanoma-involved regional lymph nodes. Lee *et al.* employed tetramers to tyrosinase, MART-1:27-35 and gp100:154-162 peptides to stain PBMC from 11 vaccine-naïve melanoma patients [103]. In four patients, MART-1-specific CTL were detected at a frequency of 0.014 - 0.16%, while tyrosinase-specific CTL were detected in two patients (0.19 and 2.2%). Based on a single patient analysis, these authors attempted to describe the status of activation of tumour reactive T-cells and concluded that the reason why cancer grows unaffected in humans is because T-cells that could potentially recognise tumour antigens are anergic. Such findings have been difficult to reproduce in studies when vaccine-induced T-cell reactivity was evaluated [101,104]. We used g209-2M tetramers to measure PBMC frequencies of vaccine-induced T-cells in patients immunised with this peptide [101]. While increases in CTL frequency could be detected in most post-immunisation PBMC, the absolute number of TA-specific T-cells reached a maximum of 1.0 - 1.5% of CD8+ T-cells. Tetramer staining appeared to correlate with other functional studies including quantitative real-time PCR and intracellular FACS analysis assessment of IFN-γ production in response to vaccine-related stimulation [104].

Several assays have been developed for measuring vaccine-induced T-lymphocyte responses. While each has advantages and disadvantages, none can measure the full spectrum of possible lymphocyte responses. In addition, there are no universally accepted correlates at this time between any method of *in vitro* immune monitoring and clinical outcome. For these reasons, these assays must be considered research techniques rather than fully validated instruments that can be used routinely to guide the development of cancer vaccines in the clinic. Monitoring becomes increasingly more directed as the antigen in the vaccine becomes more defined and restricted. In general, T-cell responses to vaccination include cell activation, changes in expression of surface markers, proliferation, cytokine production and antigen-specific lysis of target cells.

5. Functional analysis of TA-specific T-cells by gene expression

We recently developed molecular methods to estimate the presence, or changes in frequency, of T-cells in peripheral blood or tumour that have the activation characteristics in response to antigen recognition. Following activation of a T-cell, the mRNA for several cytokines and other activation-induced genes increases within 2 - 4 h as detected by quantitative RT-PCR reactions. The absolute copy number of mRNA for a particular gene and the increase in message related to T-cell activation can be measured. A sample of PBMC exposed to peptide antigen for different periods of time to can be used to study the kinetics of T-cell activation. Quantitative PCR for various cytokine genes is performed at several time points following the completion of peptide incubation [95,105].

Among several genes, IFN-γ appears to be the most sensitive for detecting activation of MHC class I-restricted CD8+ T-cells. The sensitivity of the assay, determined by spiking titrated amounts of cloned CTL to PBMC, can detect as few as one TA-specific T-cell among 50,000 PBMC. Based on preliminary studies of patients immunised with the g209-2M peptide, we believe that the sensitivity of quantitative PCR is below that of bulk *in vitro* culture of PBMC. Nevertheless the sensitivity of PCR may be sufficient for the purpose of detecting clinically relevant immune responses and the technique is substantially less labour-intensive. In addition, it may also be possible

to assess the avidity of the T-cell response by varying the concentration of peptide used to stimulate the T-cells.

6. *In vivo* biologic measures of T-lymphocyte responses

Regardless of the source of TA or method of immunisation, induction of an effective T-lymphocyte response against tumour should result in tumour infiltration by T-cells and other inflammatory effector cells drawn to the site by antigen-specific responses. Therefore, a direct method of monitoring vaccine efficacy is to obtain pre- and post-immunisation samples of tumour and assess the tumour inflammatory response. Optimally, the monitoring of T-cell responses within a tumour in response to a particular cancer vaccine would include assessment of the change in number of TA-specific cells, their activation state and functional properties. However, techniques capable of monitoring all aspects of the T-cell response are not yet available and the serial tumour biopsies necessary for these laboratory studies and full histologic examination are difficult to obtain, particularly in patients that have poorly accessible metastatic disease.

To address these concerns, we have adapted quantitative real time PCR to serially measure absolute amounts of RNA messages for genes expressed within samples of tumour obtained by FNA. While not able to ascribe changes to a particular group of antigen-specific T-cells, this technique can theoretically provide important information on changes in the overall amount of T-cell infiltration as well as the activation state and function of those cells. More importantly, the technique has the capacity to quantitatively measure biologic functions that are activated as part of a presumed final common pathway for antigen-specific T-cell mediated antitumour response. Preliminary experiments were conducted retrospectively on patients that had received g209-2M peptide vaccine [95]. In eight out of nine patients, post-treatment tumour samples revealed at least a 2-fold increase in copies of IFN-γ message; furthermore, the increase in IFN-γ mRNA correlated strongly to pre-vaccination tumour expression of gp100. Of substantial interest was the observation that none of the sampled lesions had shown evidence of objective response, suggesting that the immune response was insufficient to mediate tumour regression. The PCR

assay is currently being expanded to measure RNA message for other relevant genes, IL-2 for example.

7. Summary

The identification of TA has raised interest in the induction of specific activation and TA recognition by antigen-specific CTL. However, systemic T-cell responses to the vaccines often do not lead to objective clinical tumour regression. Among the questions raised by this paradoxical observation stands the enigma of whether tumour resistance to immunotherapy is due to insufficient immune response [106] or because tumour cells rapidly adapt to immune pressure by switching into less immuno-genic phenotypes [65]. Of note, however, the vast majority of clinical trials were performed under less than optimal circumstances and often in patients with significantly advanced cancer. Furthermore, random-ised Phase III trials with suitable control arms are only in the early stages. Despite the disappointment with epitope-specific vaccines, they have provided the unique opportunity of relating systemic T-cell responses with their localisation and status of activa-tion in the target organ. It is likely that, in the future, combined analyses of systemic and intra-tumoural immune responses with new technologies described in this review will allow a more potent understanding of the algorithm governing tumour rejection by the immune system and lead to better vaccines with improved clinical responses.

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decrease the likelihood of producing useful founder transgenic pigs that express neutralizing TGEV antibodies. Mosaicism in transgenic mice is ~35%[7] and is likely to be higher in farm species. However, the viability of the resulting transgenic offspring is likely to be normal as the transgene products should only be produced in the mammory gland during lactation.

Third, widely varying titers of recombinant Mabs were detected in the milk of the transgenic mice, suggesting wide variations in the expression of the recombinant gene in the milieu of the mammary gland. It is possible that gene rearrangement and mosaicism might affect the recombinant Mab expression in transgenic animals. Whether the transgenic mice would continue to produce the recombinant Mab in subsequent lactations also needs to be evaluated in further studies.

Fourth, the safety of this approach needs

to be further characterized in the target host species (swine), including any effects on levels of endogenous Ig production and the potential for transgenic swine to develop antibodies to the murine V_L or V_H modules of the recombinant Mab.

Finally, it is well-recognized that secretory (s)IgA antibodies produced in the milk of immune sows play a major role in lactogenic immunity to TGEV[2]. Thus, although the present studies focus on human recombinant IgG, Mabs, future work should target the use of porcine myelomas producing IgA to engineer chimeric Mabs containing the C_H and C_L of porcine IgA. The issue to address in these studies will be whether the recombinant IgA Mab expressed in mammary tissue will be dimeric and capable of interacting with the secretory component for effective secretion of sIgA into the milk.

These potential pitfalls aside, the pioneering results of Enjuanes and colleagues

provide encouragement that the production of transgenic antibodies may be a means for producing effective immunity in commercially valuable livestock. Further studies in this field, including the creation of transgenic swine secreting recombinant IgA Mab in milk capable of neutralizing TGEV and the testing of their potential to confer lactogenic immunity to TGEV in pigs, are eagerly awaited.

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Transfected human dendritic cells as cancer vaccines

Margaret Liu

Since the declaration of President Nixon's war against cancer in the 1970s, great advances have been made in the design of therapies to specifically induce or exploit immune responses against tumor cells, complementing more traditional anticancer agents that target cellular metabolism. A recent variation on this theme, pioneered in Eli Gilboa's laboratory, is to transfect dendritic cells—the professional antigen-presenting cells (APCs) of the immune system—with RNA encoding antigen and subsequently induce cytotoxic T lymphocytes (CTLs) targeted against tumors[1]. In earlier work, Gilboa's team demonstrated that transfected dendritic cells can be transferred back to animals, engendering protective immunity in mice with tumors. In this issue, they go on to show that the ability of RNA to transfect dendritic cells and stimulate CTLs in vitro extends to human cells[2]. Moreover, they demonstrate that RNA without any transfecting agent can directly transfect immature dendritic cells, but that a transfecting agent, such as a cationic lipid, is necessary to transfect more mature dendritic cells, as measured by their ability to induce CTLs in vitro.

Initial efforts to exploit the immune system to cure cancer were based on one of two premises. The first was that proteins that are expressed either uniquely or in significantly increased amounts on tumor cells can provide targets to direct "smart bombs" (or "magic bullets," as they were then called) specifically to the tumor. Types of therapeutics that fall into this category include antibodies conjugated to toxins or radioactive agents and so-called bispecific antibodies that both bind to the tumor cells and direct

other cells to kill the tumor. The second premise was that a stimulus (e.g., the administration of cytokines) could be used to boost the body's own cellular response against tumor-specific neoantigens.

As the nature of the role and mechanism of generation of CTLs became better understood—in particular for major histocompatibility complex (MHC) class I–restricted CTLs—much effort focused on devising an approach combining both of the above premises. In essence, the com-

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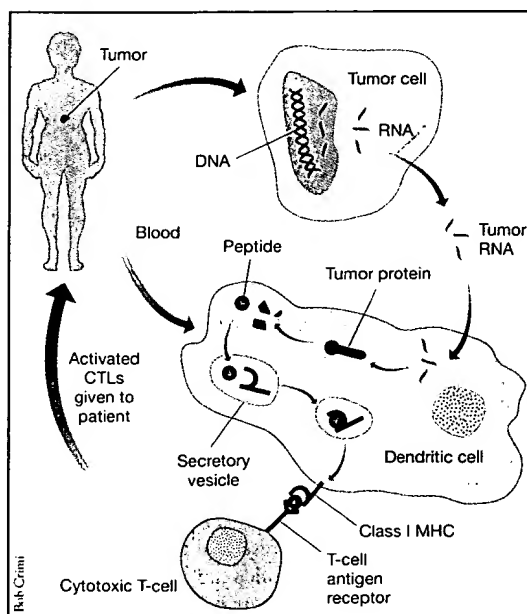
Figure 1. A hypothetical cancer immunotherapy model. Even without knowing which proteins are unique to a tumor, antigen-specific immunotherapy may be possible. RNA encoding a tumor antigen (CEA) or possibly extracted from the patient's tumor is added to dendritic cells isolated from the patient. These dendritic cells take up the RNA, even without the use of a transfecting agent, and the tumor antigen(s) is translated, entering the antigen processing pathway. Peptide derived from the tumor antigen binds to MHC class I molecules, and the dendritic cells thus prime tumor-specific CTLs in vitro when fresh peripheral blood lymphocytes are added in vitro and stimulated twice. These CTLs could then be given back to the patient.

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bined strategy aims to generate CTLs specific for tumor cells by identifying tumor-associated antigens and then to stimulate CTLs that are unique for epitopes from those proteins. (This contrasts with the use of cytokines to augment nonantigen-specific CTL responses.) The quest has been complicated somewhat by both a lack (until recently) of facile means to induce CTL and a dearth of knowledge concerning the antigens that are unique to the tumors (again an area in which significant progress has recently been made).

Several aspects of the paper by Nair et al.[2] are noteworthy. First, by using RNA derived from tumor cells, the authors circumvent the need to know which proteins the tumor is synthesizing. They also avoid the need to understand which of the proteins are specific to the tumor in order to generate dendritic cells in vitro that present epitopes of the neoantigen. Second, their approach demonstrates that human dendritic cells can be transfected by RNA without a transfecting agent (although this observation is possibly more significant for understanding the mechanism of transfection of cells than for clinical relevance). Finally, their method offers a convenient way to measure CTL activity in autologous target cells.

Several groups have shown that human dendritic cells can be used to prime CTLs using methods other than RNA transfection. Work carried out by Knight and colleagues[3] showed that human dendritic cells derived from peripheral blood could be sensitized to prime human CTLs against HIV by previous exposure to HIV in vitro. For tumor therapy, Nestle et al.[4] recently carried out a human clinical trial demonstrating that tumor lysate, or a mixture of tumor peptides, can be used to sensitize dendritic cells from patients with melanoma. When these dendritic cells were given back as a vaccine, five out of 16 patients exhibited a clinical response, including two complete responses with regression of distal metastases. These patients showed heightened cellular immune responses, including CTL activation and delayed-type hypersensitivity. As does the RNA approach of Nair et al., the tumor lysate system has the advantage of needing no prior knowledge of the antigens unique to the tumor.

There are, however, several concerns related to the RNA approach. First, because Nair et al. use cellular RNA from tumor cells, dendritic cells are transfected with RNA encoding normal as well as tumor proteins, introducing normal proteins into the same antigen processing and presentation pathways as the tumor antigen. Thus, there may be an increased risk of autoimmune phenomenon, particularly if this approach is combined with other immune-enhancing approaches such as simultaneous administration of cytokines.

Second, it remains to be demonstrated that tumor RNA-transfected dendritic cells can actually induce effective CTLs in vivo either in mice or in humans. Various cancer cells downregulate their expression of MHC class I molecules, making them poorer targets for any CTL response that is generated. Nair et al. claim that the technology is able to generate potent CTLs, but the levels of CTL killing in their paper need to be compared with killing by in vivo stimulated CTLs to determine their robustness. In addition, some of the other claims (e.g., the effect of LAMP on increasing helper T-cell responses), require more conclusive demonstration because the difference in the killing seen with various constructs is only 2–11% in nearly every case.

Importantly, only 60% of the patients with tumors expressing carcinoembryonic antigen (CEA) responded by generating CTLs when their dendritic cells were transfected. These patients could all have been primed in vivo to generate CTLs against CEA epitopes presented by their own cells, as Pardoll and colleagues[5] have demonstrated via cross-priming mechanisms[5,6].

Thus, it is not clear whether the CTLs from the five patients in this study were actually boosted memory cells, rather than in vitro primed CTLs. Given that possibility, it is perhaps more surprising that 40% of the cancer patients did not demonstrate CTL activity following the in vitro stimulation.

Lastly, it should not be assumed that all of the CTL activity shown is due to CD8^{+} MHC class I–restricted CTLs because, in most of the paper's figures, the targets were transfected with the entire CEA RNA; thus, many epitopes could be present, including MHC class II–restricted epitopes. Indeed, the authors use killing of such RNA-transfected dendritic cells by CD4^{+} to illustrate their claim regarding the induction of T-cell help. The amount of killing of irrelevant targets in some cases may indicate that natural killer cells were induced.

These reservations aside, the technique used by Nair et al. may greatly facilitate CTL studies in outbred species and represent a valuable means of inducing tumor-specific CTL responses for cancer immunotherapy, possibly obviating the need to identify specific tumor antigens. It is unknown whether this represents a more useful technique than sensitization of dendritic cells with tumor lysate[4] or harnessing the ability of dendritic cells to take up apoptosed cells[6]. Whatever the case, these observations do provide insight into dendritic cell differentiation and the cellular uptake of polynucleotides.

The latter may be important for increasing the potency of nucleic acid vaccines, wherein plasmid DNA or in vitro transcribed RNA versions encoding antigens have been shown to induce immune responses when directly injected in vivo without any transfecting agent. At present, DNA rather than RNA is the molecule of choice for inducing immune responses (including potent CTL responses based on precursor frequency studies) because of its stability and proven capability to protect in a variety of preclinical models[7].

For tumor immunotherapy by in vitro transfection of dendritic cells, however, RNA provides a means to capitalize on the tumor cells' own regulation of protein production in an effort to induce cellular immune responses directed against the tumor.

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... poorer efficacy due to immunoselection and appearance of antigen-negative clones within the tumor. Novel approaches to vaccine design using gene transfection with cytokines and *dendritic* *cells* are all promising. However, the induction of immune responses does not necessarily confer a therapeutic benefit. Therefore, these elegant newer strategies need to be studied...

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Tumor-associated antigens as tools in immunodiagnostics and immunotherapy of breast cancer
    EINSATZ TUMORASSOZIIERTER ANTIGENE IN DER IMMUNTHERAPIE UND
IMMUNDIAGNOSTIK DES MAMMAKARZINOMS

    Guckel B.; Meuer S.; Bastert G.; Wallwiener D.
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    ...of antigen-defined vaccines. Direct in vivo administration of peptides in combination with adjuvants suitable for establishing effective immune responses or ex vivo loading of *dendritic* *cells* with tumor-specific epitopes are target-specific immunation approaches. Cocktails of synthetic peptide epitopes should permit targeting *multiple* *antigens* while avoiding the development of antigen-loss variants. Most clinical phase I/II trials to date indicate that cellular or peptide-based vaccines are safe...
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         44449    DENDRITIC(W)(CELL OR CELLS)
        830430    DIFFERENTIAL
        154739    DISPLAY
         85146    SCREEN
        529513    SCREENING
         12372    DIFFERENTIAL(W)((DISPLAY OR SCREEN) OR SCREENING)
     S3     37     (DENDRITIC (W) (CELL OR CELLS)) AND (DIFFERENTIAL (W)
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        1074138   MULTIPLE
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Albert B. DeLeo[3],
Michael T. Lotze[1, 2] and
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Genetically modified bone marrow-derived dendritic cells expressing tumor-associated viral or "self" antigens induce antitumor immunity in vivo

The clinical application of synthetic tumor peptide-based vaccines is currently limited to patients with specified major histocompatibility complex (MHC) class I alleles. Such logistic limitations may be overcome using tumor gene-based approaches. Here we describe the effective generation of dendritic cells (DC) expressing tumor peptide-MHC complexes as a result of particle-mediated transfer of genes encoding tumor-associated antigens (TAA). Bone marrow-derived DC were transfected with plasmid DNA encoding the tumor-associated viral antigen E_7 derived from human papilloma virus (HPV) 16. When applied as a vaccine, these genetically modified DC induced antigen-specific CD8+ cytotoxic T lymphocytes (CTL) in vivo and promoted the rejection of a subsequent normally lethal challenge with an HPV 16-transformed tumor cell line. Of greatest interest, immunization of mice with syngeneic DC genetically modified to enhance their presentation of a constitutive "self" epitope derived from the tumor-suppressor gene product p53 caused a significant reduction in the in vivo growth of a chemically induced p53-positive sarcoma. These results suggest that cancer vaccines consisting of DC genetically modified to express TAA of viral or "self" origin effectively induce antitumor immunity in vivo.

1 Introduction

CTL play a crucial role in the host's immune response to cancer. The adoptive transfer of tumor-specific CTL can mediate the regression of established tumors in experimental animal models [1], as well as, in some patients with melanoma [2]. Recently the molecular basis of CTL-mediate tumor immunity has been elucidated. A number of genes encoding tumor-associated antigens and their peptide products, which are recognized by CTL in the context of major histocompatibility complex (MHC) class I molecules, have been identified for both murine and human tumors [3, 4]. The translation of these insights into the development and application of novel immunotherapies is one of the principal challenges for contemporary tumor immunologists.

DC are believed to be critical for the induction of primary, cell-mediated immune responses [5, 6]. Using freshly isolated, as well as, cultured DC pulsed with peptides constituting relevant CTL-defined epitopes, we and others have induced protective and therapeutic antitumor immune responses in murine tumor models [7–13]. The translational potential of this approach has been demonstrated in vitro using autologous cultured human DC pulsed with synthetic melanoma peptides, which are able to stimulate

antigen-specific "self"-reactive CTL capable of lysing HLA-matched allogeneic melanoma cells that naturally express these epitopes [14–16]. As an alternative to synthetic peptides that may restrict the immune response to defined tumor-associated epitopes with known MHC restriction, the use of plasmid DNA or recombinant viruses encoding tumor-associated antigen (TAA) has recently been investigated for the immunotherapy of cancer [17–23]. The direct insertion of genes encoding known TAA into immunostimulatory DC, resulting in the endogenous production and processing of relevant antigenic peptides, is an area of active current research [24–30]. We report here our initial study using a non-viral, particle-mediated gene transfer technology [31–34] in order to express plasmid DNA encoding TAA in cultured murine bone marrow-derived DC. These genetically modified DC process and present CTL-defined epitopes derived from tumor-associated viral or "self" antigens, and mediate protective antitumor immunity in vivo in two well-characterized murine tumor model systems.

2 Materials and methods

2.1 Mice, cell lines, and antibodies

Female C57BL/6 mice, 6–10 weeks old, were obtained from Taconic (Germantown, NY). Female BALB/cJ mice, 6–10 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained in a specific pathogen-free facility (Central Animal Facility, University of Pittsburgh). C3 is a human papillomavirus (HPV) 16-transformed murine sarcoma [35]. CMS4 and MethA are chemically induced BALB/c sarcomas [12]. EL4 was obtained from the American Type Culture Collection (Rockville, MD). Culture supernatants containing mAb produced by the following hybridomas obtain from the ATCC were used in this study: anti-L3T4 (TIB207), anti-Lyt-2.2 (TIB210), and anti-B220 (TIB146). All cell

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lines were maintained in culture medium (CM) consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM Hepes, 5 μM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin and were determined to be free of mycoplasma contamination (Geneprobe, Fisher Scientific, Pittsburgh, PA). Anti-mouse MHC Class II, anti-mouse B7.1, and anti-mouse B7.2 mAb were purchased from Pharmingen (San Diego, CA) and used in standard flow cytometric assays for phenotypic analysis.

2.2 Peptides and plasmids

The H-2D^b-binding peptide E$_7$ (aa49–57, RAHYNIVTF) derived from HPV16-E$_7$ [35] and the H-2K^d-binding peptide p53 (aa232–240wt, KYMCNSSCM) derived from wild-type murine p53 were synthesized by standard Fmoc chemistry and purified by HPLC in the Peptide Synthesis Facility of the University of Pittsburgh Cancer Institute (shared resource). The plasmid pCMV-lux containing a firefly luciferase gene was kindly provided by Geniva (Madison, WI). The plasmid pEGFP containing a variant of the green fluorescent protein gene was purchased from Clontech (Palo Alto, CA). The plasmid pCI-E$_7$ was constructed by subcloning the ORF of HPV16-E$_7$ into the expression plasmid pCI (Promega, Madison, WI) using PCR-techniques. The plasmid pCI-p53(aa225–285)wt was constructed by subcloning bp 675 to 855 of the mouse p53 cDNA previously isolated from the CMS4 sarcoma [12] flanked by an artificial start and stop codon to allow for translation of the resulting minigene into the expression plasmid pCI using PCR-techniques. All inserts were sequenced in both directions to exclude mutations introduced by PCR. Plasmids were grown in *E. coli* strain DH5α and purified using Qiagen Endofree Plasmid Maxi Kits (Qiagen, Chatsworth, CA).

2.3 Generation of DC and particle-mediated gene transfer

DC were prepared from bone marrow as previously described [12] with minor modifications. Briefly, bone marrow cells were harvested from femurs and tibias, immunodepleted of lymphocytes by treatment with anti-B220, -CD4, and -CD8 mAb followed by rabbit complement (Accurate, Westbury, NY), and cultured (37°C, 5% CO$_2$) in 6-well plates at 5 × 10^5 cells/3 ml/well in CM supplemented with 1000 U/ml each of murine rGM-CSF and rIL-4 (Schering-Plough, Kenilworth, NJ). Cells were depleted of loosely adherent granulocytes on day 2 and 4 and fed with fresh CM containing rGM-CSF and rIL-4. Loosely adherent cells were harvested on day 8 by gentle pipetting. Fifty to seventy percent of DC routinely expressed the surface markers MHC class II, B7.1, and B7.2 as measured by flow cytometry. Plasmid DNA was precipitated onto 0.95-μm gold particles at a density of 2 μg of DNA per mg of particles as previously described [34]. Briefly, gold particles and DNA were resuspended in 100 μl of 0.05 M spermidine (Sigma Chemical Co., St. Louis, MO) and DNA precipitated by the addition of 100 μl of 1 M CaCl$_2$. Particles were washed in dry ethanol to remove H$_2$O, resuspended in dry ethanol containing

0.075 mg/ml of PVP (Sigma), and coated onto the inner surface of Tefzel tubing using a tube loader. The tubing was cut into 0.5-inch segments resulting in the delivery of 0.5 mg gold coated with 1 μg plasmid DNA per transfection with the Accell helium pulse gun. Gold particles, tubing, tube loader, and the Accell helium pulse gun were kindly provided by Geniva (Madison, WI). Bone marrow-derived DC were transfected in suspension in 6-well plates. DC were harvested, pelleted by centrifugation, 2 × 10^6 cells resuspended in 20 μl of fresh medium, spread evenly in the center of a prewetted well, bombarded, and fresh CM was added immediately.

2.4 Assays for expression of luciferase and green fluorescent protein

Expression of luciferase was determined using an Autolumat LB 953 (eG&G Berthold). Transfected cells were washed with Hanks' balanced salt solution (HBSS, GIBCO-BRL), lysed in 100 μl cell culture lysis reagent (Promega), and stored at −80°C. Samples were thawed, cell debris pelleted, and 10 μl of cell extract assayed in duplicate with Luciferase Assay System (Promega). The level of sample luminescence was recorded as relative light units (RLU). Experiments were performed three times. To assess transfection of individual dendritic cells, expression of green fluorescent protein was determined by fluorescent microscopy. Transfected cells were cultured on sterile fibronectin-coated cover slips for 24 h. Images were taken using standard FITC fluorescent filters on a Zeiss Axiovert with a Photometrics (Tuscon, AZ) 12-bit cooled CCD camera. Fluorescent and differential interference contrast (DIC) images were subsequently processed using BDS image software (Biological Detection Systems, Pittsburgh, PA).

2.5 Immunization of mice and generation of cytotoxic effector populations

Following particle-mediated gene transfer, DC were irradiated (3000 rad), washed twice with HBSS, and 5 × 10^5 cells in 0.5 ml HBSS injected in the tail vein of syngeneic mice on days 0 and 7. On day 14 mice were injected with 2 × 10^6 C3 tumor cells subcutaneously in the left flank or with 1.5 × 10^5 *in vivo* expanded CMS4 tumor cells subcutaneously in both flanks. Tumor growth was assessed every 3 to 4 days and recorded as tumor area (mm^2) by measuring the largest perpendicular diameters. Fisher's exact method or Student's *t*-tests were performed to interpret the significance of differences between experimental groups (presented as mean ± SEM). All experiments include five mice per group and were performed three times. Spleens from immunized mice or from nonimmunized littermates were harvested and pooled on day 14 and restimulated with naive peptide-pulsed splenocytes. Stimulator cells were incubated with peptides for 3 h at 37°C (10 μg peptide/10^7 cells/ml), washed twice in HBSS, and irradiated (3000 rad) before use. Responder cells (10^6) were cultured with 3 × 10^6 stimulator cells per well of a 24-well plate in CM for 5 days (37°C, 5% CO$_2$). Cytotoxic effector cells (5 × 10^5) were restimulated weekly with 4 × 10^4 irradiated (10 000 rad) C3 tumor cells and 3 × 10^6 irradiated (3000 rad) splenocytes as feeder cells per well of

a 24-well plate in CM containing 25 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA) to obtain long-term CTL lines recognizing the E_7 (aa49–57) peptide.

### 2.6 Cyt toxicity and cytokine release assays

In vitro restimulated lymphocytes were tested for their cytolytic reactivity against EL4 cells ± E_7-peptide in triplicate in standard 4-h ^{51}Cr-release assays using 96-well round-bottom plates. Target cells (2×10^6) were radiolabeled with 100 µCi Na_2-$^{51}CrO_4$ (NEN-Dupont, Bedford, MA) for 1 h at 37°C. Peptide-pulsed targets were prepared by incubating cells with peptide at a concentration of 1 µg/ml in CM for 30 min at room temperature prior and 0.5 µg/ml during the cytotoxicity assay. Maximum and spontaneous, as well as the percentage of specific ^{51}Cr-release, were determined by standard procedures [12]. CTL were washed twice before use. Cytokine release assays were performed in duplicate using 96-well round-bottom plates. Peptide-pulsed DC (2.5×10^4; prepared by incubation with 1 µg/ml peptide for 1 h at 37°C) or 10^5 TAA-transduced DC were used as stimulators and 2.5×10^4 cells of a long-term CTL line recognizing the E_7 (aa49–57) peptide were used as responders per well. Supernatants were harvested after 24 h and murine IFN-γ content was measured by sandwich ELISA using purified rat anti-mouse IFN-γ (clone R4-6A2, Pharmingen) at a concentration of 2 µg/ml as a capture antibody and biotinylated rat anti-mouseIFN-γ (clone XMG1.2, Pharmingen) at a concentration of 1 µg/ml as a detection antibody. The detection limit of this assay was 30 pg/ml of rmIFN-γ (Pharmingen).

3 Results

3.1 Rapid transgene expression following particle-mediated gene transfer to DC

A firefly luciferase gene was used as a sensitive reporter gene in order to optimize the parameters for particle-mediated gene transfer to DC using the Accell helium pulse gun. Expression of luciferase was monitored over a 8–72-h time course (Fig. 1a). Significant levels of transgene were detected within 8 h, with maximal levels occurring 16–24 h after transfection. DC transfected with an irrelevant plasmid produced only background levels of bioluminescence. Bombardment of 2×10^6 DC with 1 µg plasmid DNA coated onto 0.5 mg of gold particles delivered at a pressure of 300 psi of helium resulted in the highest transgene expression, while maintaining > 75 % of the cell viability vs. mock-transfected cultures and was used routinely.

To detect transgene expression on a single cell basis, the novel reporter gene enhanced green fluorescent protein (EGFP) derived from the bioluminescent jellyfish Aequorea victoria was used. Expression of EGFP can be detected by fluorescence microscopy without the aid of developing antibodies or substrates since the EGFP chromophore derives from the primary protein sequence itself. DC with brightly fluorescent cytoplasm outlining the typical veiled morphology due to cytoplasmic expression of EGFP could be observed 24–48 h after particle-mediated gene transfer
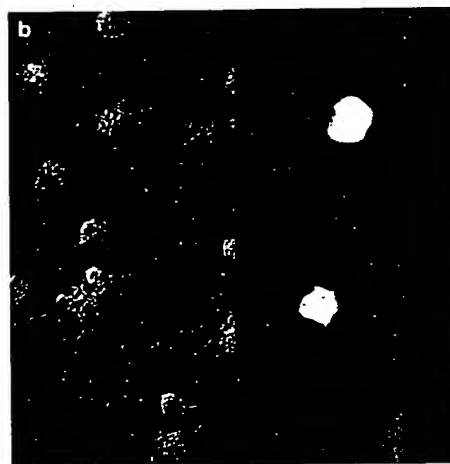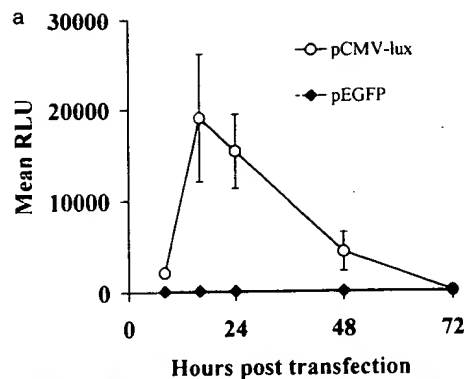
Figure 1. Expression of firefly luciferase and EGFP after particle-mediated gene transfer to DC. (a) DC were lysed 8–72 h following particle-mediated gene transfer of expression plasmids encoding firefly luciferase (pCMV-lux) or EGFP (pEGFP). Maximal levels of sample bioluminescence were detected 16–24 h after transfection of DC with pCMV-lux but not with pEGFP. (b) Alternatively, DC were imaged 24 h after transduction using standard FITC fluorescent filters (left) and differential interference contrast (right). Cytoplasmic fluorescence of DC displaying a typical veiled morphology could readily be detected after transduction with pEGFP but not with pCMV-lux. Typically 5–10 % of DC were transfected using this approach.

(Fig. 1b) with an estimated frequency of 5–10 % of the total number of cells expressing the transgene. The majority of EGFP-transduced DC displayed high levels of MHC class II and B7.2 molecules, as measured by simultaneous staining with PE-conjugated mAb and flow cytometry.
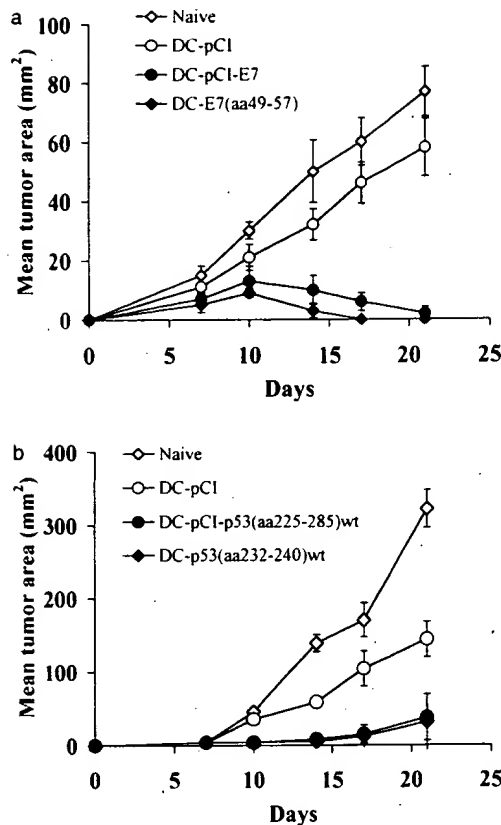
3.2 DC genetically modified to express TAA stimulate peptide-specific CTL and promote protective antitumor immunity in vivo

Particle-mediated gene transfer of pCl-E_7, an expression plasmid encoding the tumor-associated viral antigen HPV16-E_7, into cultured DC resulted in the secondary stimulation of a CTL line recognizing the H-2D^b-restricted peptide E_7 (aa49–57) as evidenced by antigen-specific cytokine release (Fig. 2a). This demonstrates that transduced DC were able to express, process, and present T cell epitopes derived from the TAA in an immunogenic format.
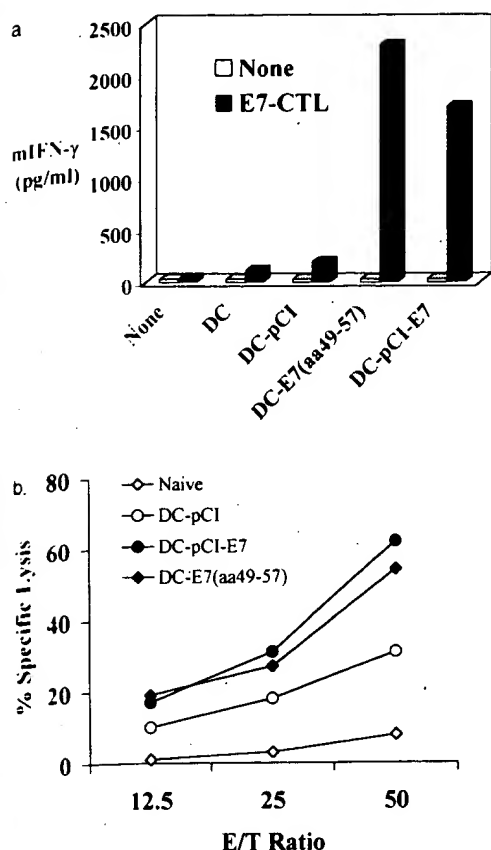
Figure 2. TAA-transduced DC stimulate antigen-specific memory CTL *in vitro* and induce primary antigen-specific CTL *in vivo*. (a) Twenty-four hours following transduction of DC with pCI-E7, a plasmid encoding the viral TAA HPV16-E7, expression, processing, and presentation of the known D^b-binding peptide-epitope derived from aa49-57 was assessed in a standard cytokine release assay using antigen-specific long-term CTL lines. pCI-E7-transduced or E7 (aa49-57) peptide-pulsed DC elicited significantly higher levels of cytokine release compared to control-transduced or unpulsed DC. ELISA results from a representative experiment of three are shown. (b) Cytolytic activity against EL4 tumor cells sensitized with the E7 (aa49-57) peptide could be detected in splenocytes harvested from mice immunized with pCI-E7-transduced or E7 (aa49-57) peptide-pulsed DC after *in vitro* restimulation.

Figure 3. DC genetically modified to express the viral TAA HPV-E7 or the self-antigen p53 mediate protective antitumor immunity *in vivo*. (a) Immunization of mice with DC transduced with pCI-E7 or pulsed with the E7 (aa49–57) peptide lead to rejection of a subsequent, normally lethal challenge with the HPV16-transformed tumor cell line C3. All animals tumor free at day 21 remained tumor free through day 90. (b) Immunization of mice with DC transduced with pCI-p53(aa225–285)wt, a minigene construct expressing aa225–285 of wild-type murine p53, or pulsed with the K^d-binding peptide derived from aa232–240 of wild-type p53 significantly inhibited the *in vivo* growth of the CMS4 sarcoma expressing a mutated p53 protein (missense mutation at codon 194) which accumulates in the tumor and leads to enhanced presentation of the wild-type peptide aa232–240 on H-$2K^d$. At the termination of these experiments (day 90), 60 % of animals in both the DC-pCI-p53 and the DC-p53 peptide immunized groups were tumor free.

The efficacy of a vaccine consisting of DC genetically modified to express HPV16-E7 was then assessed *in vivo*. pCI-E7 was inserted into DC by particle-mediated gene transfer and 5 × 10⁵ cells were injected intravenously into C57BL/6 mice on days 0 and 7 as a vaccine. This resulted in the induction of CTL recognizing the E7 (aa49-57) peptide pulsed onto EL4 tumor cells (Fig. 2b). Reactivity against unpulsed EL4 target cells was similar to controls. We have repeatedly observed unusually high background cytolytic activity against both peptide-pulsed and unpulsed EL4 cells when using cultured DC as stimulators *in vivo*. This has been previously reported by another group [10] and is most likely due to immune responsiveness to MHC-presented, FBS-derived determinants extracted from the DC culture medium. Importantly however, mice immunized with PCI-E7-inserted or E7-peptide-pulsed DC were protected against a challenge on day 14 with the C3 sarcoma, an HPV16-transformed cell line latently expressing E7 [35], while naive mice or mice immunized with DC

transfected with pCI control plasmid did not reject the tumor challenge (Fig. 3a).

We also examined whether DC genetically modified to express a tumor-associated "self" antigen could mediate antitumor immunity. The CMS4 sarcoma accumulates high levels of p53 molecules due to a missense mutation at codon 194 of p53. This leads to enhanced natural processing and presentation of the H-$2K^d$-binding "self"-epitope p53(aa232–240)wt, a potential CTL target [12]. pCI-p53(aa225–285)wt, a plasmid DNA minigene construct expressing aa225–285 of the wild-type sequence of murine p53, was inserted into DC by particle-mediated gene transfer and 5 × 10⁵ cells were injected intravenously into BALB/c mice on days 0 and 7 as a vaccine. This also resulted in the induction of antigen-specific CTL (data not shown). Upon challenge with CMS4 on day 14, a significant reduction of tumor growth could be observed in the

DC-pCI-p53 and the DC-p53 peptide-immunized groups compared to DC-pCI immunized or naive mice (Fig. 3b).

4 Discussion

In the past, gene-based strategies for the immunotherapy of cancer have targeted the genetic modification of tumors in order to increase their inherent immunogenicity. However, it has become increasingly evident that the efficient induction of tumor-specific CTL responses requires that the relevant antigenic peptides be presented to T cells by professional host antigen-presenting cells (APC) [36]. The ability to generate large numbers of immunostimulatory DC *in vitro* from precursor populations in bone marrow and blood using GM-CSF and IL-4 [37–41] has stimulated great interest in the development of vaccines consisting of adoptively transferred DC which are loaded with tumor antigens *ex vivo*. Here we demonstrate the introduction of genes encoding TAA into DC. While the use of synthetic peptides requires prior knowledge of the patients HLA haplotype, as well as, the relevant T cell epitopes, gene-based vaccines lead to the intracellular expression of the entire TAA in the APC. This allows for the concurrent processing and presentation of multiple, clinically important but, as yet, undefined MHC-restricted epitopes. Although the use of viral vectors may be more efficient for gene transfer into DC [26, 29, 30], non-viral gene delivery methods have important advantages: (1) more than one gene can be transfected simultaneously allowing co-transfection of several antigens along with genes encoding immunostimulatory cytokines; (2) immune responsiveness to xenogeneic vector-derived immunogens is minimized; (3) they utilize purified DNA, which can readily be produced in large quantities, is very stable, and bears little risk of recombination.

Our results indicate that particle-mediated gene transfer of plasmid DNA encoding defined TAA of viral or "self" origin directly into murine bone marrow-derived DC generates APC that can induce antigen-specific CTL, resulting in protective antitumor immunity *in vivo*. Both tumor models investigated are clinically relevant. More than 90 % of human cervical carcinoma harbor human papillomavirus, predominantly HPV16. HPV16, as well as other "high-risk" HPV, are considered to play an important role in the pathogenesis of human cervical cancer. CTL epitopes have been identified for several HLA haplotypes, with active specific immunotherapy being pursued for prevention and treatment of cervical carcinoma [42]. Furthermore, a large number of human cancers accumulate p53 protein due to missense mutations in the p53 gene. This frequently results in the enhanced presentation of MHC class I-associated peptides, predominantly derived from nonmutated ("wild-type") portions of the p53 molecule. Indeed, wild-type p53 epitopes have been identified, that are recognized by HLA-A2-restricted CTL *in vitro* [43, 44]. Such peptide-reactive CTL are also able to recognize p53 positive human tumor cells [45–47]. These results support the potential clinical utility of p53 epitopes in tumor vaccines.

The gene transfer method presented here allows us to investigate now in greater detail the qualitative and quantitative nature of the antitumor immune response elicited by vaccines consisting of genetically modified *vs.* peptide-pulsed DC. Several limitations of this potentially useful approach (*i.e.* the induction of unwanted autoimmune responses, or, conversely, of immunologic tolerance) need to be carefully assessed. Furthermore, since DC-based vaccine approaches necessitate costly and time-consuming *ex vivo* cell cultures, comparisons will have to be performed against DNA-based immunization targeting skin or muscle, which may be technically easier to apply in the clinic. These latter routes of administration are dependent upon uptake of antigen by tissue-resident APC, either through direct transfection of APC or indirectly via the reprocessing of antigens released from alternate transfected cells [48–51]. Additionally, defective APC function *in situ* has been described in tumor-bearing animals, which may be overcome by short term *ex vivo* culture of bone marrow precursors [52]. We therefore anticipate that the use of cultured DC as a biological adjuvant for DNA-based cancer vaccines will considerably enhance the induction of antitumor immune responses.

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   Identification and immunocytochemical analysis of DCNP1, a *dendritic*
cell-associated nuclear protein.
   Masuda Masako; Senju Satoru; Fujii Si Shin-ichiro; Terasaki Yasuhiro;
Takeya Motohiro; Hashimoto Si Shin-ichi; Matsushima Kouji; Yumoto Eiji;
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   Identification and immunocytochemical analysis of DCNP1, a *dendritic*
cell-associated nuclear protein.
   Dendritic *cells* (DCs) are potent antigen-presenting cells (APCs).
Among so-called professional APCs, only DCs can activate naive T cells to
initiate immune response. To better understand molecular mechanisms
underlying unique functions of DCs, we searched for genes specifically
expressed in human DCs, using PCR-based cDNA subtraction in conjunction
with *differential* *screening* . cDNAs generated from CD34(+) stem
cell-derived CD1a(+) DC were subtracted with cDNA from monocytes and used
for generation of a cDNA library. The cDNA...

...expressed in DCs more abundantly than in monocytes. We identified a gene
encoding a protein composed of 244 amino acids, which we designated as
DCNP1 (*dendritic* *cell* nuclear protein 1). In Northern blot analysis,
DCNP1 mRNA was highly expressed in mature DCs and at a lower level in
immature DCs. In contrast, monocytes and B cells do not express the gene.
In *multiple* human tissue Northern blot analysis, expression of DCNP1 was
detected in brain and skeletal muscle. To examine subcellular localization
of DCNP1, we performed immunofluorescence analysis...
   Descriptors: *Dendritic* *Cells*--immunology--IM; *Nuclear Proteins
--genetics--GE; Amino Acid Sequence; Antigens, CD--analysis--AN; Antigens,
CD34--analysis--AN; Antigens, Differentiation, Myelomonocytic--analysis--AN
; Base Sequence; Cells, Cultured; Cloning, Molecular; *Dendritic* *Cells*
--chemistry--CH; Gene Library; Immunohistochemistry; Immunophenotyping;
Microscopy, Fluorescence; Molecular Sequence Data; Monocytes--chemistry--CH
; Nuclear Proteins--analysis--AN; Nuclear Proteins--immunology--IM; RNA,
Messenger--biosynthesis...
   Chemical Name: Antigens, CD; Antigens, CD34; Antigens, Differentiation,
Myelomonocytic; CD68 antigen, human; Nuclear Proteins; RNA, Messenger;
dendritic *cell*-associated nuclear protein 1
?ds

Set   Items   Description
S1   5   (DENDRITIC (W) CELL?) (S) ((MULTIPLE (W) ANTIGENS) OR (ARR-
                 AY (W) OF (W) ANTIGENS))
S2   3   RD (unique items)
S3   37   (DENDRITIC (W) (CELL OR CELLS)) AND (DIFFERENTIAL (W) (DIS-
                 PLAY OR SCREEN OR SCREENING))
S4   3   S3 AND (POLYEPITOPES OR ARRAY OR MULTIPLE)
S5   1   RD (unique items)
?s s3 and (immunotherapy or cancer or tumor)
            37   S3
        114792   IMMUNOTHERAPY
       2236483   CANCER
       2112670   TUMOR
    S6   6   S3 AND (IMMUNOTHERAPY OR CANCER OR TUMOR)

Human Dendritic Cells Genetically Engineered to Express a Melanoma Polyepitope DNA Vaccine Induce Multiple Cytotoxic T-Cell Responses[1]

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ABSTRACT

Purpose: To assess the therapeutic potential of a melanoma polyepitope vaccine in human cells. Polyepitope DNA vaccines encoding T-cell epitopes have been demonstrated in murine systems to generate multiple cytotoxic T-cell responses to different antigens. Here, for the first time we demonstrate the ability of a melanoma polyepitope to stimulate lymphocytes from normal human donors to simultaneously generate multiple antigen-specific responses.

Experimental Design: Human dendritic cells (DC), transduced with a melanoma-polyepitope cDNA, were used to activate autologous lymphocytes from naïve donors as an *in vitro* model of DNA vaccination. Lymphocytes were primed with polyepitope or mock-transduced DC, boosted with peptide, then measured for antigen-specific cytotoxicity.

Results: Lymphocytes primed with polyepitope-transduced DC and boosted with peptide generated multiple cytotoxic responses. By contrast lymphocytes primed with mock-transfected DCs and boosted with peptide gave no specific cytotoxicity. However, when lymphocytes were repeatedly stimulated with polyepitope-transduced DCs immunodominance was seen with CTLs being generated to only one epitope, MART$_{27-35}$.

Conclusions: We show in a human system that a melanoma polyepitope primes CTL to multiple epitopes. However, repeated stimulation by the polyepitope restricts the response to only the MART1 epitope. Thus, although polyepitope vaccines are an effective way of priming multiple naïve T-cell responses, continual boosting with polyepitope vaccines may, as a result of immunodominance, restrict the CTL. These findings have important implications for the use of DNA polyepitope vaccines in cancer immunotherapy.

INTRODUCTION

The discovery of melanoma-associated antigens has resulted in a proliferation of clinical trials based on antigen- and epitope-specific immunization against cancer. Protocols have used direct injection of peptide epitopes with adjuvant (1) or alternatively, DCs[3] pulsed with peptide epitopes and readministered to patients (2). Although peptide injection is logistically easier, adoptive transfer of *ex vivo* pulsed DCs may provide more potent activation signals to CTLs, avoiding their deletion attributable to presentation by nonprofessional adenomatous polyposis coli (3). This is largely because DCs possess high levels of MHC and costimulatory molecules necessary for potent activation of helper and cytotoxic T cells (4). Although CTL responses are elicited in patients immunized by both methods, these are often not associated with significant clinical regression of tumors, and many mechanisms have been described by which tumors can evade immune responses (5). Down-regulated expression of antigen by tumor cells is one of the more effective ways of evading an epitope-restricted CTL response. Strategies that use several antigens can circumvent this, and the polyepitope approach (encoding multiple MHC class I-restricted epitopes as one cDNA) is one such example (6). Studies in mice revealed that when virally vectored polyepitopes were administered, CTL responses were elicited despite the lack of natural flanking sequences (7–9). Therefore, this approach allows epitopes from different antigens to be administered simultaneously, reducing the antigen loss evasion mechanisms of tumors. Polyepitopes also negate the need to vaccinate with whole, potentially oncogenic DNA sequences (10). In addition polyepitopes can be delivered as plasmid DNA and injected directly without the need for viral vectors, and can incorporate signal sequences that improve processing or include MHC class II-restricted helper epitopes (11–13).

To date, work with polyepitope vaccines for cancer has focused on murine models. In human leukocyte antigen-A2 transgenic mice, responses vary from 50% to 100% of epitopes (14–16). However, the immune repertoire and antigen-processing machinery in man varies significantly from that of mice. Importantly, as many melanoma antigens are "self-antigens"

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tolerance may prevent the successful use of these in vaccines in man, despite apparent efficacy in mice. Cultured DCs have been used to test human immune responses *in vitro* and, when modified with peptide, DNA, or recombinant virus, induce CTL responses from naïve lymphocytes (17). Responses to a single epitope from influenza virus were described when a polyepitope incorporating this epitope was tested in retrovirally transduced DCs (18). However, many individuals have been exposed previously to influenza, and so this may not be an ideal model for a tumor epitope (19).

We designed a polyepitope to encode multiple melanoma-associated epitopes restricted to HLA-A1 and HLA-A2. When human lymphocytes were primed with polyepitope-transduced DCs and boosted with peptide, multiple CTL responses were seen. However, repeated boosting to polyepitope-transduced DCs resulted in a single dominant CTL response to an epitope from the MART-1/Melan-A melanoma antigen. This may have implications for the scheduling of polyepitope DNA vaccination and DC-based vaccines in humans.

MATERIALS AND METHODS

Polyepitope Construction. The polyepitope sequence (296 bp) was synthesized using four oligonucleotides (Iain Goldsmith, ICRF Oligonucleotide Synthesis Service) that overlapped by 20 bp and together covered the full polyepitope sequence. The cDNA was assembled by PCR using the splicing by overlap extension technique (9). Briefly, adjacent primers were dimerized in separate PCR reactions after which primer dimers were spliced together to generate the full-length sequence. Products from the oligonucleotide splicing by overlap extension steps were used as templates for a final PCR amplification reaction using 20-bp terminal primers. Gel-purified polyepitope cDNA (termed poly-MEL) was restricted using *Bam*HI and *Eco*RI and cloned into the pVAX1 expression vector (Invitrogen). Constructs were sequenced to verify their fidelity.

Transfection of COS7 Cells. Before transfection (24 h), COS7 cells were seeded into six-well plates (Costar; 2.5×10^5 cells/well) in DMEM (Life Technologies, Inc.) supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin (Life Technologies, Inc.). Transfections were carried out using 2.5 μg of pVAX1 vector or pVAX-poly-MEL with 15 μl of DOTAP reagent (Boehringer Mannheim) for immunofluorescence or with 12 μl of LipofectAMINE reagent (Life Technologies, Inc.) for Western blotting in 1 ml of medium for 5 h. After transfection (24 h), cells were trypsinized, transferred to glass slides, and cultured for an additional 48 h. Alternatively, transfectants for lysis and Western blotting were cultured continuously in six-well plates for 72 h.

Indirect Fluorescence Confocal Microscopy. After transfection (72 h), cells were fixed with 3.7% formaldehyde/PBS and then permeabilized in 0.1% Triton-X-100/PBS. Permeabilized cells were probed with anti-pk antibody (Serotec) in PBS and detected with Alexa-Fluor 488-conjugated goat anti-mouse antibody (Molecular Probes Inc.) and confocal fluorescence microscopy (Leica TCS-SP). Cell cytoskeleton and nuclei were stained with Phalloidin conjugated to Alexa-Fluor 594

(Molecular Probes Inc.) and 4′,6-diamidino-2-phenylindole, respectively, and slides mounted in Mowiol 4–88 (Calbiochem).

Western Blotting. Transfected cells were resuspended in disruption buffer [5% glycerol, 50 mм Tris-HCl (pH 7.0), 2% SDS, 5% 2-mercaptoethanol, and 0.25% bromphenol blue] and stored at −20°C. After boiling for 5 min, samples were electrophoresed on a 16% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham) using the Novex Xcell II blotting apparatus. After blocking in Tris-buffered saline with 0.1% Tween plus 10% skimmed milk the membrane was incubated with anti-pk antibody. Bound antibody was detected using biotinylated rabbit antimouse (Dako), horseradish peroxidase-conjugated streptavidin (Dako), and enhanced chemiluminescence (Amersham).

Recombinant Adenovirus Containing Poly-MEL. Recombinant adenovirus was synthesized using the AdEasy system (20). The pAdEasy1 adenoviral backbone vector, the pAdTrack-CMV shuttle plasmid, and the *Escherichia coli* strain BJ5183 were gifts from Dr. Tong-Chuan He (John Hopkins Oncology Center, Baltimore, MD). The 293 packaging cell line was obtained from ICRF Central Cell Services and maintained in DMEM supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin (Life Technologies, Inc.).

Poly-MEL was subcloned into the pAdTrack-CMV shuttle plasmid using the *Bam*HI and *Eco*RI restriction sites. Electrocompetant BJ5183 were then cotransformed with 1 μg of *Pme*I linearized pAdTrack-CMV/poly-MEL and 0.1 μg of supercoiled pAdEasy1. Plasmids obtained from ampicillin-resistant colonies were screened for recombinant pAd-poly-MEL vectors, which were subsequently amplified in DH5α *E. coli* (Life Technologies, Inc.). Control virus was generated using "empty" pAdTrack-CMV vectors. Cells (293) were transfected with 4 μg of *Pac*I linearized pAd-poly-MEL or pAd-empty and 20 μl of LipofectAMINE reagent/25 cm² flask (Costar). After 7 days, cells were harvested and lysed by four cycles of freeze/thaw/vortexing to obtain viral supernatants. Adenovirus was amplified by repeated infection of 293 cells, and virus supernatants were stored at −20°C. The inclusion of the green fluorescent protein gene in pAdTrack vectors was used to estimate viral titers by infecting 293 cells and counting green fluorescent cells by fluorescence microscopy after 24 h.

HLA-A2 Typing. Aliquots of PBMC from buffy coats of healthy donors were tested with the anti-HLA A2 antibody BB7.2 (ICRF Research monoclonal antibody service), the binding of which was detected by FITC-conjugated goat antimouse (Dako) and flow cytometry.

Generation of MD-DCs. Buffy coats obtained from healthy blood donors were provided by the National Blood Service (Seacroft Hospital, Leeds, United Kingdom). PBMC were isolated by centrifugation of diluted buffy coats through Lymphoprep (Nycomed). After washing, cells were transferred to six-well plates (Costar) at 2×10^7 PBMC/well in RPMI 1640 with 2% heat-inactivated fetal calf serum, L-glutamine, and penicillin/streptomycin, and incubated for 1.5 h at 37°C. The nonadherent fraction were harvested and frozen, and the adherent cells were cultured for 6 days in 3 ml/well of DC medium (RPMI 1640/10% HI-FCS supplemented with 800 units/ml granulocyte macrophage colony-stimulating factor (Leucomax; Schering Plough) and 500 units/ml IL-4 (R&D Systems).

Medium (1 ml) was replaced with fresh medium plus cytokines on day 3. On day 6 immature MD-DCs were harvested as nonadherent cells.

Adenoviral Transduction of MD-DCs. Adenovirus infection was carried out by incubation of 10^6 immature MD-DCs in 1 ml of adenoviral supernatant (multiplicity of infection = 100) for 2 h at 37°C. Transduced DCs were washed and transferred to a 24-well plate at 10^6 cells/ml of DC medium including 1000 units/ml of TNFα (R&D Systems) for 24 h to induce maturation.

In Vitro Generation of CTL Responses. TNFα matured, 7-day-old, transduced MD-DCs were irradiated (3000 rads) and mixed with autologous PBLs that comprised the nonadherent fraction of PBMC used to generate MD-DCs. Cells were combined to give 10^5 MD-DCs and 2×10^6 PBL/ml of CTL medium [RPMI 1640, 7.5% heat-inactivated human serum type AB (Sigma Chemical Co.), L-glutamine, penicillin/streptomycin, HEPES, nonessential amino acids, sodium pyruvate (Life Technologies, Inc.), and 200 μM 2-mercaptoethanol (Sigma Chemical Co.)] plus 5 ng/ml of IL-7 (R&D systems). Cells were distributed into 24-well plates (Costar) at 1 ml/well and cultured at 37°C. After 3 days and as required thereafter, cultures were fed with CTL medium plus 5 ng/ml IL-7. IL-2 (Proleukin) was added at this stage to a final concentration of 20 IU/ml. On day 7 and weekly thereafter, responding PBL were restimulated with autologous PBMC pulsed separately with peptide epitopes (Dr. Nicola O'Reilly, ICRF Peptide Synthesis Laboratory) corresponding to the A2-restricted epitopes from poly-MEL. For pulsing, PBMC were resuspended at 10^6 cells/ml in serum-free RPMI 1640 with L-glutamine and penicillin/streptomycin. Peptide was added to a final concentration of 50 μg/ml after which pulsing was carried out at 37°C with occasional agitation of cell suspensions. After 4 h, PBMC were washed, irradiated (3000 rads), and cocultured with responder PBL at a ratio of 1:1 in CTL medium with IL-7. Alternatively PBL primed in week 1 with adenovirally transduced MD-DCs were restimulated weekly with fresh poly-MEL transduced MD-DCs as described above.

Cytotoxicity Assays. Responding PBL were tested for specific cytotoxicity in chromium release assays. T2 cells (174xCEM.T2 hybridoma, transporter associated with antigen presentation-1-, and transporter associated with antigen presentation-2-deficient; American Type Culture Collection) were simultaneously labeled with of [^{51}Cr]sodium chromate (Amersham) at 100 μCi/10^6 cells and peptide-pulsed with 10 μg/ml of each melanoma epitope, influenza matrix epitope($_{58-66}$) or with no epitope. After three washes, target cells were mixed with responder PBL so the killing by each population was tested against targets loaded with relevant, control and no peptide. "Cold" K562 cells (no MHC expression and sensitive to natural killer cell-mediated lysis) were included at 50 × the target cell number to inhibit nonspecific lysis. Assays (4-h) were carried out in 96-well plates with 5000 targets/well in a final volume of 200 μl RPMI 1640 with 10% FCS. All of the E:T ratios were tested in triplicate. Spontaneous release was determined in medium alone and maximal release in 1% Triton-X-100 (Sigma Chemical Co.). Chromium release was assayed by liquid scintillation using a 1450 MicrobetaJet (Wallac), and percentage-specific cyto-

Table 1 Tumor antigens and related epitopes included in the poly-MEL polyepitope

Tumor antigen[a] (epitope position)	Epitope sequence (HLA restriction)	Score[b]
gp100 (154–162)	KTWGQYWQV (A2)	316
gp100 (280–288)	YLEPGPVTA (A2)	1
MAGE-1 (161–169)	EADPTGHSY (A1)	250
MAGE-3 (161–169)	EVDPIGHLY (A1)	250
MAGE-3 (271–279)	FLWGPRALV (A2)	2655
Melan-A/MART-1 (27–35)	AAGIGILTV (A2)	2
Tyrosinase (1–9)	MLLAVLYCL (A2)	309
Tyrosinase (368–376)	YMDGTMSQV (A2)	213

[a] Refs. 22–27.
[b] Estimated half time of disassociation, Ref. 21.

toxicity was calculated using the following formula: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100.

Staining with Fluorogenic HLA-Tetramer. Cells were stained in phosphate buffered saline with phycoerythrin-labeled MART-1 tetramer at 37C for 20 min, washed at room temperature, and incubated on ice with antibodies to CD8 (CD8-PerCP; BD Biosciences).
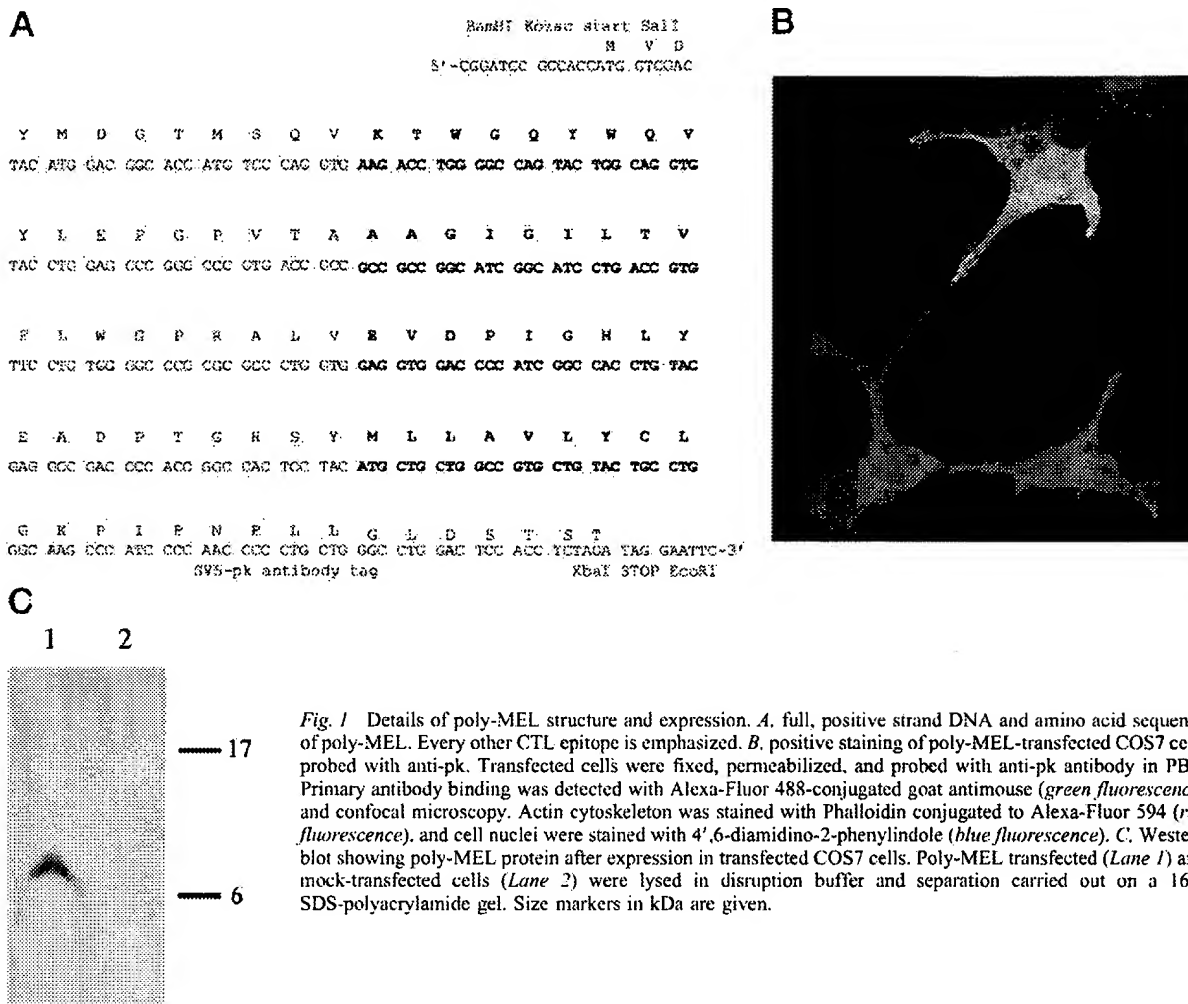
Statistics and Epitope Binding Predictions. Analysis of cytotoxicity data were performed using two-tailed Student's *t* tests assuming equal variance. Epitope binding predictions were obtained using an algorithm based on Parker *et al.* (21).

RESULTS

Polyepitope: Poly-MEL. Eight epitopes described previously, known to be recognized on the surface of melanoma cells by CTL, were chosen for inclusion in poly-MEL (Table 1). Only epitopes restricted to HLA-A1 or HLA-A2 were included, because these two alleles are common in melanoma-prone populations. Epitopes were directly linked without flanking sequences in the poly-MEL sequence, which included a Kozac consensus start sequence, the SV5-pk antibody tag, a stop codon, and restriction sites to facilitate future modifications (Fig. 1A). Human optimized codon usage was used, and unavoidable internal start codons were positioned to minimize initiation of translation.

When transfected COS-7 cells were probed using anti-pk antibody, staining of the cytoplasm was seen in pVAX1/poly-MEL-transfected cells (Fig. 1B). In addition, Western blotting revealed a specific protein corresponding with the predicted mass, absent in mock-transfected cells (Fig. 1C). Thus, recombinant pol-MEL protein is synthesized by transfected cells. This is in agreement with the results of Hanke *et al.* (12), who similarly used the pk tag to successfully label an HIV polyepitope. The poly-MEL sequence was then expressed in an adenoviral system for efficient delivery to MD-DC. We have shown this system previously to potently transduce DC without perturbing their maturation or function (28). Despite Western blotting, confocal microscopy, and flow cytometry, we have not detected the polyepitope in adenoviral-transduced DC.

Polyepitope-transduced DC Prime Multiple CTL Responses. To measure induction of T-cell responses in a human system, *in vitro* stimulation of lymphocytes from four

A

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                              BamHI Kozac start SalI
                                       M   V   D
                              5'-CGGATCC GCCACCATG.GTCGAC
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Y   M   D   G   T   M   S   Q   V   K   T   W   G   Q   Y   W   Q   V
TAC ATG GAC GGC ACC ATC TCC CAG GTG AAG ACC TGG GGC CAG TAC TGG CAG GTG
```

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Y   L   E   F   G   P   V   T   A   A   G   I   G   I   L   T   V
TAC CTG GAG CCC GGC CCC GTG ACC CCC GCC GCC GGC ATC GGC ATC CTG ACC GTG
```

```
F   L   W   G   P   R   A   L   V   E   V   D   P   I   G   H   L   Y
TTC CTG TGG GGC CCC CGC GCC CTG GTG GAG GTG GAC CCC ATC GGC CAC CTG TAC
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E   A   D   P   T   G   H   S   Y   M   L   L   A   V   L   Y   C   L
GAG GCC GAC CCC ACC GGC CAC TCC TAC ATG CTG CTG GCC GTG CTG TAC TGC CTG
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G   K   P   I   P   N   P   L   L   G   L   D   S   T   S   T
GGC AAG CCC ATC CCC AAC CCC CTG CTG GGC CTG GAC TCC ACC TCTACA TAG GAATTC-3'
        SV5-pk antibody tag                         XbaI STOP EcoRI
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B

C


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1   2
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— 17

— 6

Fig. 1 Details of poly-MEL structure and expression. *A*, full, positive strand DNA and amino acid sequence of poly-MEL. Every other CTL epitope is emphasized. *B*, positive staining of poly-MEL-transfected COS7 cells probed with anti-pk. Transfected cells were fixed, permeabilized, and probed with anti-pk antibody in PBS. Primary antibody binding was detected with Alexa-Fluor 488-conjugated goat antimouse (*green fluorescence*) and confocal microscopy. Actin cytoskeleton was stained with Phalloidin conjugated to Alexa-Fluor 594 (*red fluorescence*), and cell nuclei were stained with 4',6-diamidino-2-phenylindole (*blue fluorescence*). *C*, Western blot showing poly-MEL protein after expression in transfected COS7 cells. Poly-MEL transfected (*Lane 1*) and mock-transfected cells (*Lane 2*) were lysed in disruption buffer and separation carried out on a 16% SDS-polyacrylamide gel. Size markers in kDa are given.

HLA-A2+ donors was carried out. Poly-MEL or mock-transduced DC were used to prime autologous lymphocytes for 1 week. Transduced DC displayed high levels of CD40, CD80, and MHC class II, which were up-regulated after exposure to TNFα (data not shown), confirming that DC were not adversely affected by adenoviral transduction. After priming, responder lymphocytes were "boosted" separately in week 2 and thereafter with autologous PBMC pulsed with peptide. Cytotoxicity assay demonstrated that multiple CTL responses were generated from lymphocytes primed with poly-MEL-transduced DC (Fig. 2). CTL for all six of the HLA-A2-restricted epitopes were elicited from 1 donor (Fig. 2B). Responses to 5 of 6 epitopes were seen in two donors (Fig. 2, C and D), whereas in donor A, were generated to three epitopes. No cytotoxicity was seen toward influenza matrix peptide loaded T2 targets or of unloaded T2 cells. By contrast lymphocytes stimulated by mock-transfected DCs and restimulated with peptide generated no epitope specific CTLs. (Fig. 2, mock prime). These results demonstrate that all six of the HLA-A2-restricted epitopes from poly-MEL

could be processed by human DCs and presented to autologous T lymphocytes from naïve donors. Furthermore, this allowed priming of the corresponding CTL responses from PBL, which were measurable after expansion in the presence of peptide epitopes.

The frequency of MART-1-specific T cells was determined by staining unstimulated and primed/boosted lymphocytes with tetrameric-HLA-A2/MART-1. Two of four donors had detectable MART-1-specific CTL before stimulation. Activation and expansion of CTL by poly-MEL resulted in a significant increase in the proportion of CD8+ cells stained with the MART-1 tetramer (Fig. 3). This was observed for all four of the donors.

Restricted CTL Induction by Repeated Stimulation with Polyepitope. To determine whether poly-MEL could prime and boost multiple CTL responses in human lymphocytes, autologous lymphocytes were repeatedly stimulated with poly-epitope or mock-transduced DC. In all four of the donors, after three rounds of restimulation, specific killing was only seen of MART-1$_{27-35}$ loaded targets. (Fig. 4). Even after five rounds of
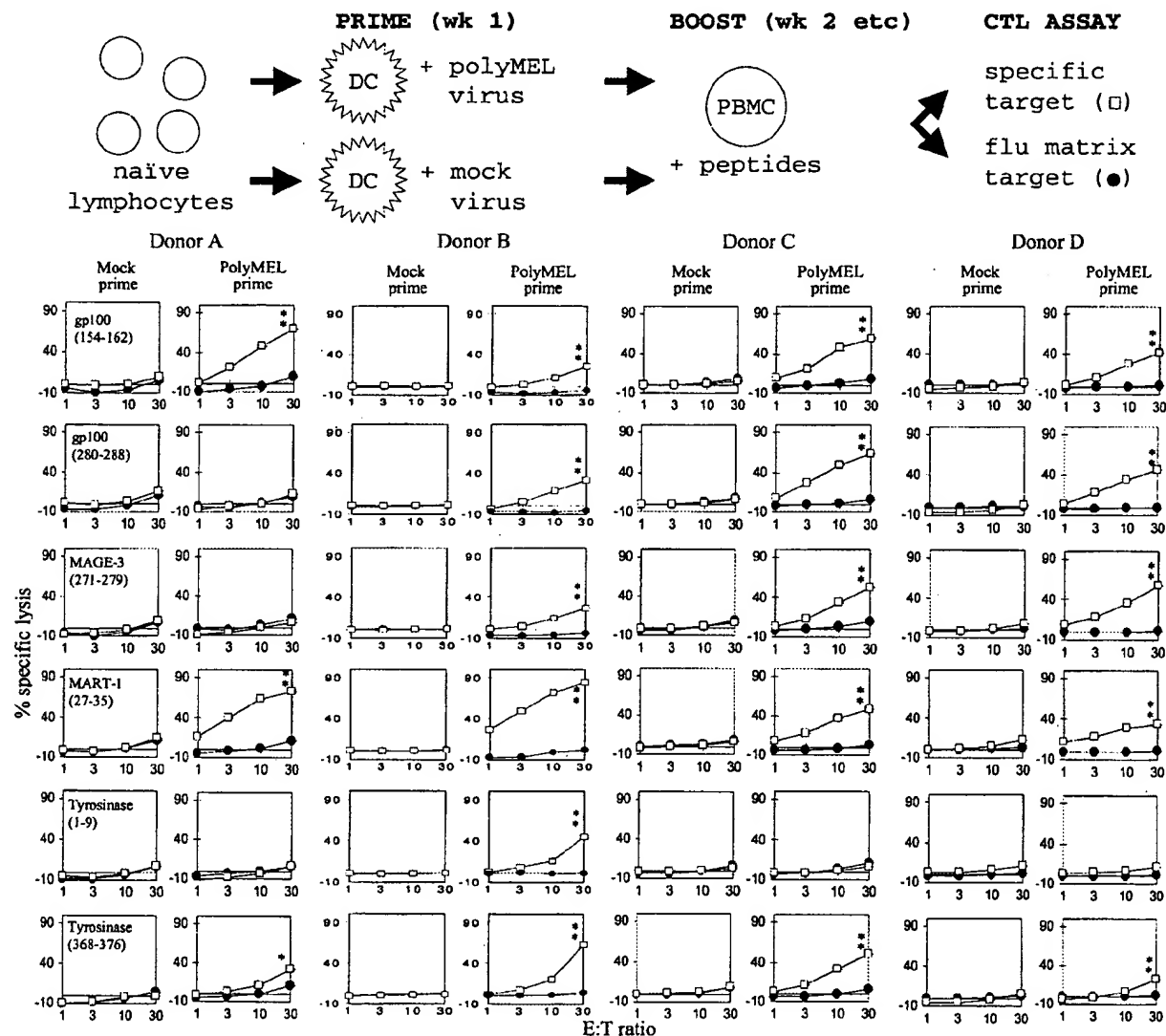
Fig. 2 Multiple CTL responses induced by poly-MEL. CTL responses after MD-DC priming followed by restimulation with autologous, peptide-pulsed PBMC as shown in cartoon form. Responder PBL were primed in week 1 with either poly-MEL or mock-transduced MD-DC as indicated. From week 2 onwards, polyepitope and mock-primed cells were split and restimulated using PBMC pulsed with the peptide epitope indicated in the first graph of each row. Killing of [51]chromium-labeled T2 cells was tested for each responder population after three restimulations. Targets were pulsed with either the specific peptide epitope indicated for each row (□) or with flu matrix (58–66) epitope (●). Each point represents the mean and SD of triplicate experiments. Significant differences in cytotoxicity against specific and control targets at the 30:1 E:T ratio are indicated (** *P* < 0.001, * *P* < 0.005). Representative results from four donors are given.

stimulation with polyepitope-transduced DC, it was not possible to generate any other CTL specificities (data not shown). These findings illustrate that despite the ability to prime CTL to all of the HLA-A2-restricted epitopes, repeated stimulation with poly-epitope-transduced DC induced CTL responses to 1 of 6 epitopes, namely MART-1$_{27-35}$.

DISCUSSION

This study sought to address the therapeutic potential of a polyepitope vaccine designed to induce multiple CTL responses against melanoma antigens. These included the tu-

mor-specific antigens MAGE-1 and MAGE-3 as well as differentiation antigens gp100, tyrosinase, and MART-1. Expression of tumor-specific and differentiation antigens has been described for a large proportion of patients with melanoma with heterogeneous expression of gp100 and MART-1 but consistent tyrosinase expression on all of the melanoma-associated metastatic lesions examined (29). In addition, MAGE-1 and MAGE-3 are expressed in 30–70% patients with metastatic melanoma illustrating the potential value of multivalent vaccines. We have shown that our polyepitope is translated into protein and can prime responses to the major-
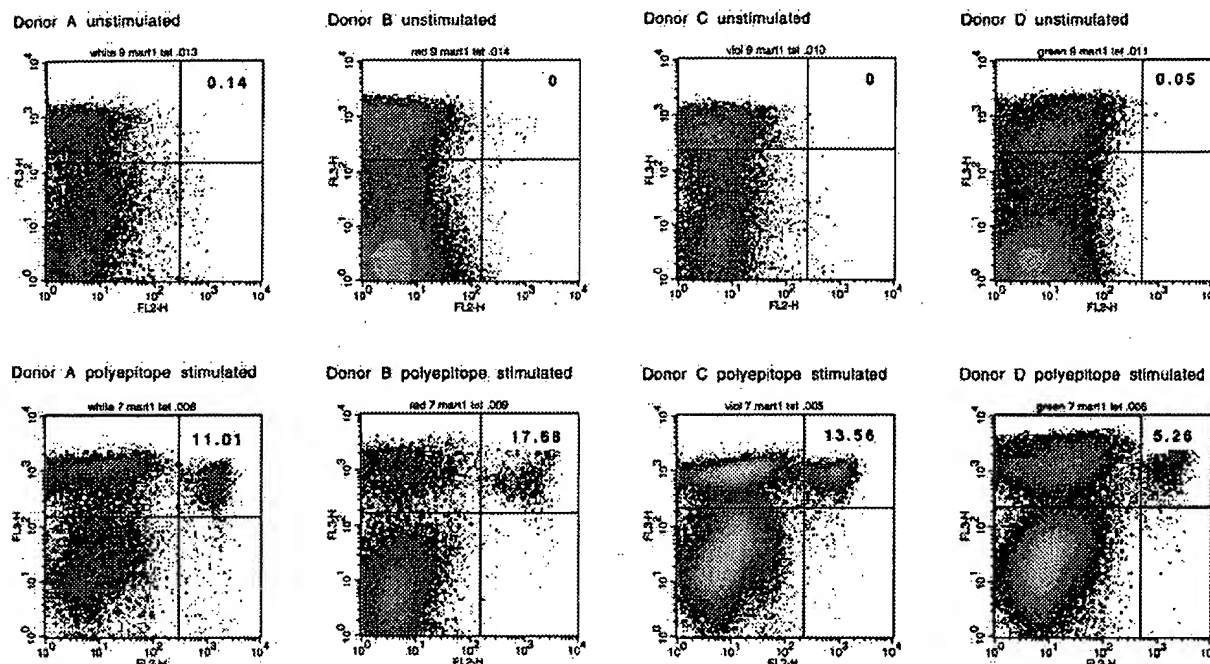
Fig. 3 Staining of unstimulated and poly-MEL stimulated lymphocytes with MART-1-specific tetrameric HLA-A2. Freshly derived lymphocytes (*ex vivo*) and CTL taken from cultures primed and boosted with DC/poly-MEL were stained for MART-1-reactive T-cell receptor using fluorogenic HLA-A2-tetramers and counterstained with anti-CD8. The results are from four donors (*A–D*), and the percentage of tetramer-positive CD8+ T cells is shown in each plot.

ity of epitopes encoded but that its repeated use can lead to the generation of an immunodominant response.

Polyepitope protein was shown to be translated in agreement with others (12). However, despite generating immune responses some other groups have not detected polyepitope proteins (8, 14). In our study although polyepitope could be found in COS7 cells, we did not see it in virally transduced DC (data not shown). We have observed large variations previously in the abundance of different polyepitope proteins in transfected cells.[4] Variation in polyepitope abundance may reflect differences in mRNA stability or be a consequence of protein stability. This may relate to the ability of different polyepitope proteins to form secondary and tertiary structures and, hence, resist proteolytic processes within the cell (8).

Our study demonstrates priming of CTL responses specific for up to 6 of 6 HLA-A2 epitopes by transduced human DC. To our knowledge, this is the first report of multiple CTL responses induced by a polyepitope in a human system. Heemskerk *et al.* (18) used human CD34+-derived DC transduced with retrovirally vectored polyepitope and demonstrated a single CTL response to the influenza matrix$_{(58-66)}$ epitope. In an HLA-A2 transgenic murine model, 4 of 7 and 7 of 10 A2-restricted epitopes induced CTL responses after vaccination with HIV and melanoma polyepitopes, respectively (14, 16). These experiments involved polyepitope

priming and boosting *in vivo* followed by splenocyte stimulation *in vitro* with peptide-sensitized blast cells, thus paralleling the *in vitro* experiments in our study. Although these experiments used DNA constructs encoding several antigens that are incorporated in poly-MEL, several differences were seen. The polyepitope used by Mateo *et al.* (16) failed to induce any responses against the tyrosinase$_{1-9}$ or the MAGE-3$_{271-279}$ epitopes. In contrast, in human cells responses were seen in 1 of 4 and 3 of 4 donors in our study. This may relate to the inability of HLA-A2 transgenic mice to respond to some epitopes because of a limited T-cell repertoire or to differences in TAP processing. Indeed, vaccination of HHD mice with either tyrosinase$_{1-9}$ or the MAGE-3$_{271-279}$ peptides also failed to elicit CTL (16). Alternatively this may be in part attributable to differences in the two polyepitope constructs. Thus, though sophisticated transgenic mice provide invaluable preclinical models for testing such therapeutic approaches, studies in a fully human system can give key preclinical data not available from murine models.

Multiple stimulation with poly-MEL-transduced DC led to immunodominance with generation of CTLs to only one epitope. The reasons for this are unclear but as suggested by Yewdell and Bennink (30), may include binding of peptide to HLA, effect of precursor frequency, more efficient liberation of dominant epitopes by DCs, and suppression of T-cell reactive to subdominant epitopes by those specific for immunodominant epitopes. The affinity of epitope for HLA is unlikely to simply account for the immunodominance, because the predicted affin-

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[4] Unpublished observations.
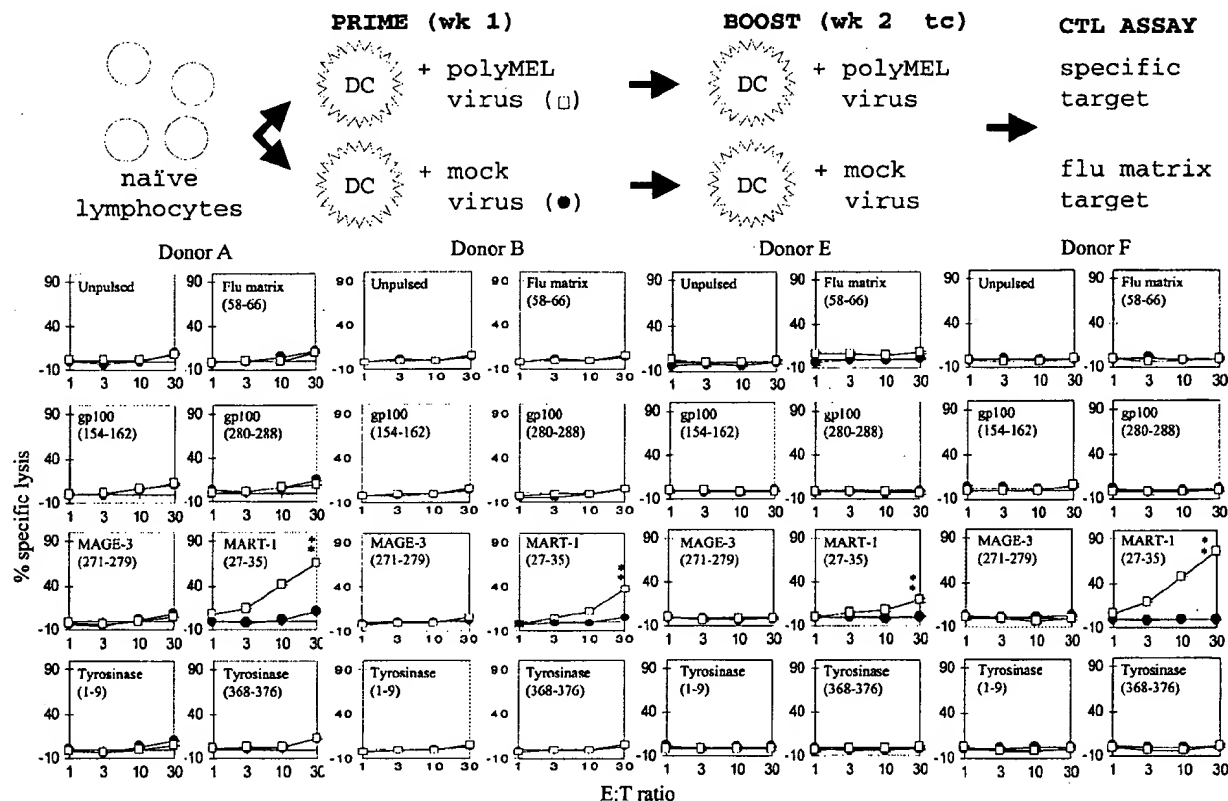
Fig. 4 Repeated stimulated with poly-MEL leads to immunodominance. CTL responses after repeated stimulation of autologous PBL with transduced MD-DC, shown in cartoon form. Responder PBL received up to 5 weekly stimulations with irradiated, poly-MEL- (□) or mock- (●) transduced MD-DC. Responder lymphocytes were tested for specific cytotoxicity against [51]chromium-labeled T2 cells pulsed with the indicated peptide epitope. Each point represents the mean and SD of triplicate experiments. Significant differences in cytotoxicity mediated by polyepitope and control induced effectors at the 30:1 E:T ratio are indicated (** $P < 0.001$). Representative results from four donors are given.

ity of the MART epitope for HLA-A2 is lower than most other HLA-A2-binding epitopes in poly-MEL. Although a full study of precursor frequency of specific CTLs was not undertaken with our donors, the *ex vivo* PBL of two of four donors had a demonstrable precursor population of MART-1-reactive CD8+ T cells. Furthermore, a large proportion of HLA-A2+ healthy donors (60%) are known to possess high frequencies of MART-1-specific CTLs (31). Our own data[5] confirmed this observation, showing that 3 of 10 normal HLA-A2+ donors have a significant proportion of MART-1-specific CD8+ cells as judged by reactivity with MART-1 tetramer. By contrast, no precursors to the other antigens in our polyepitope were detected by specific tetramers. Polyepitope responses have been investigated in the presence of large numbers of preexisting CTL populations (32) and indicated that strong, existing responses inhibited vaccine-induced responses that were restricted by the same MHC haplotype. This is in agreement with the observation that a high-frequency precursor number is a contributory mechanism for immunodominance of some epitopes (33). Impor-

tantly, the abundance of professional antigen presenting cells may contribute to immunodomination as T cells compete for antigen displayed by adenomatous polyposis coli (34). Kedl *et al.* (34) demonstrated competition between high-affinity OVA-specific transgenic OT1 T cells and either subdominant OVA epitopes or unrelated epitopes. In this instance, introduction of large numbers of additional DC overcame the immunodominance by OT1 cells. It remains to be determined whether the abundance of DCs in our model *in vitro* system was responsible for the observed immunodominance.

There is a possibility that the quantity of epitope presented by polyepitope-expressing DCs is directly related to the MART-1 immunodominance observed. A previous study described an immunodominant HLA-A11-restricted CTL epitope from the EBNA3B protein of EBV that is more abundant than a related, subdominant epitope (35). Although the MART-1 epitope is predicted to bind with a much lower affinity to HLA-A2 than all but one of the A2-restricted epitopes in poly-MEL, it may be liberated from the full length polyepitope protein much more efficiently and, thus, achieve a greater availability for loading of HLA molecules than the other epitopes. Although quantitation of epitope abundance could be achieved after elution of epitopes from the MHC molecules of poly-

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[5] Unpublished observations.

epitope-expressing cells, it should be noted that immunodominance does not necessarily correlate with epitope abundance on the cell surface. It was recently demonstrated by Crotzer *et al.* (36) that the immunodominant EBV-derived epitope RRIYD-LIEL is in fact the least abundant of four EBV-derived epitopes presented by HLA-B27.

In conclusion, we have described a polyepitope vaccine designed to induce CTL responses to commonly expressed melanoma antigens. We demonstrated that the majority of HLA-A2 restricted epitopes are processed and presented by human DC and effectively prime CTL responses *in vitro*. However, as the response was restricted to a single epitope after repeated polyepitope stimulation, vaccination strategies may need to consider the relative immunodominance of epitopes included in polyepitopes and vaccination schedules. These questions are to be addressed in a DNA vaccination clinical trial in patients with malignant melanoma.

ACKNOWLEDGMENTS

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 7/3,K/1   (Item 1 from file: 155)
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14307614   22362283   PMID: 12472671
   Analysis of gene expression during maturation of immature *dendritic* *cells* derived from peripheral blood monocytes.
   Matsunaga T; Ishida T; Takekawa M; Nishimura S; Adachi M; Imai K; et al
   First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan.
   Scandinavian journal of immunology (England)   Dec 2002, 56 (6) p593-601, ISSN 0300-9475   Journal Code: 0323767
   Document type: Journal Article
   Languages: ENGLISH
   Main Citation Owner: NLM
   Record type: Completed

   Analysis of gene expression during maturation of immature *dendritic* *cells* derived from peripheral blood monocytes.
   Dendritic *cells* (DCs) are the most important antigen-presenting cells. Many recent studies have compared the function of immature DCs (iDCs) and mature DCs (mDCs), but there...

   ... during maturation. Here, we report on differential gene expression in iDCs generated from peripheral blood monocytes compared with mDCs. Gene expression was evaluated using the *differential* *display* method after activation of iDCs with a low concentration of lipopolysaccharide (LPS) to induce maturation. Proteasome subunit alpha type 3 (PSMA3), transcription factor EC (TFEC...
   Descriptors: *Dendritic* *Cells*--immunology--IM; *Monocytes--immunology--IM; *Stem Cells--immunology--IM; Apoptosis; Cell Differentiation; Cells, Cultured; Cysteine Endopeptidases--metabolism--ME; *Dendritic* *Cells* --classification--CL;   *Dendritic*   *Cells*--drug   effects--DE;   Gene Expression Profiling; Immunophenotyping; Kinetics; Lipopolysaccharides --pharmacology--PD; Multienzyme Complexes--metabolism--ME; Nerve Tissue Proteins--biosynthesis--BI; Nerve Tissue Proteins--genetics--GE...

   ...Oncogene Proteins c-bcl-2--biosynthesis--BI;   Proto-Oncogene Proteins c-bcl-2--genetics--GE;   RNA,   Messenger--biosynthesis--BI;   Transforming Growth Factor beta--pharmacology--PD; *Tumor* Necrosis Factor--pharmacology --PD
   Chemical Name: Lipopolysaccharides; Multienzyme Complexes; Nerve Tissue Proteins; Proto-Oncogene Proteins c-bcl-2; RNA, Messenger; Transforming Growth Factor beta; *Tumor* Necrosis Factor; neuronal apoptosis inhibitory protein; Cysteine Endopeptidases; multicatalytic endopeptidase complex

 7/3,K/2   (Item 2 from file: 155)
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10189008   22220530   PMID: 12235260
   Dual mechanisms for ethanol-induced inhibition of monocyte chemotactic protein-3 mRNA expression in activated glial cells.
   Ren Liqiang; Syapin Peter J
   Department of Pharmacology, Alcohol and Brain Research Laboratory, Texas Tech University Health Sciences Center, Lubbock 79430, USA.
   Journal of pharmacology and experimental therapeutics (United States) Oct 2002, 303 (1) p265-72, ISSN 0022-3565   Journal Code: 0376362
   Contract/Grant No.: AA11643; AA; NIAAA
   Document type: Journal Article

Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA

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Dendritic cells (DC) generated from the peripheral blood mononuclear cells of healthy individuals or from cancer patients transfected with carcinoembryonic antigen (CEA) mRNA stimulate a potent CD8⁺ cytotoxic T lymphocyte (CTL) response in vitro. DCs are effectively sensitized with RNA in the absence of reagents commonly used to facilitate mammalian cell transfection. RNA encoding a chimeric CEA/LAMP-1 lysosomal targeting signal enhances the induction of CEA-specific CD4⁺ T cells, providing a strategy to induce T-help that may be necessary to generate and/or maintain an optimal CD8⁺ CTL response in vivo. CEA RNA-transfected DCs also serve as effective targets in cytotoxicity assays, thus providing a general method for inducing, as well as measuring, CEA-specific CTL responses across a broad spectrum of HLA haplotypes.

The dendritic cell (DC) network is a specialized system for presenting antigen to naive or quiescent CD4⁻ and CD8⁺ T cells[1]. Numerous studies have documented the exceptional ability of DCs to stimulate T-cell responses in vitro and in vivo[2-4]. Vaccination of mice with DCs pulsed with antigens in the form of peptides or proteins is capable of priming cytotoxic T lymphocyte (CTL) responses and engender tumor immunity[9-14]. Murine DCs transfected with chicken ovalbumin (OVA) mRNA stimulate primary CTL responses in vitro and treatment of tumor-bearing animals with DCs pulsed with tumor-derived RNA leads to a reduction in lung metastases[15].

One advantage of using RNA over peptides as antigen is that RNA encodes multiple epitopes for many HLA alleles. Hence, RNA transfected DCs can be used to stimulate CTL responses in many patients without prior knowledge of, or need to determine, the haplotype of the patient. Another potentially important advantage of using RNA as a source of tumor antigens is that sufficient amounts of antigen can be generated from very small amounts of tumor tissue via amplification techniques, thus expanding the use of RNA-pulsed DC-based vaccines to patients bearing very small tumors.

To determine whether vaccination with human DCs transfected with RNA encoding antigen represents a useful strategy for cancer immunotherapy, we tested whether RNA transfected DCs could present antigen to class I-restricted CD8⁺ T cells. The antigen-presenting cell (APC) function of RNA-transfected DCs was assessed by measuring the induction of a primary CTL response against a specific tumor-associated antigen, CEA. CEA is a 180 Kda glycoprotein and is expressed on most adenocarcinomas of the gastrointestinal tract, on 50% of breast cancers, and 70% of non-small-cell lung cancers[16]. Induction of primary human CTL responses using monocytes or B-cells as APCs has been described and requires multiple cycles of stimulation with the APC[17,18]. Human DC pulsed with pep-

tides or transduced with retroviruses encoding a tumor-associated antigen were effective in generating CTL responses in vitro[19-23]. Thus, induction of primary CTL responses in vitro will constitute a stringent measure for the APC function of the RNA-transfected DCs and will provide the experimental foundation for clinical assessment of this form of tumor vaccination. We show that DCs generated from the peripheral blood mononuclear cells (PBMC) of healthy individuals or cancer patients transfected with in vitro transcribed CEA RNA or total RNA isolated from CEA-expressing tumor cells, stimulate a potent, primary, CD8⁺ CTL response in vitro.

Results

Induction of primary CTL responses using DCs transfected with RNA encoding the CEA antigen. Murine DCs transfected with RNA encoding antigen are highly effective in stimulating primary CTL responses in vitro and elicit protective immunity in tumor-bearing animals[15]. To test whether human DCs can be similarly sensitized with RNA encoded antigen, we measured the ability of DCs transfected with RNA encoding the CEA antigen to induce a primary CTL response in vitro. CEA RNA was generated in vitro by transcription of a cDNA plasmid (IVT CEA RNA). Incubation of PBMCs from an HLA-A2, CEA⁻ individual with IVT CEA RNA-transfected DCs stimulated a CEA-specific, primary CTL response as judged by the lysis of CEA peptide, but not influenza virus matrix (M1) peptide, pulsed T2 cells (Fig. 1).

Incubation of PBMCs with the M1 peptide-pulsed DCs induced CTLs capable of lysing M1 peptide, but not CEA peptide, pulsed targets. CEA RNA-transfected DCs were as effective as CEA peptide-pulsed DCs in stimulating a primary CTL response. The potency of the RNA-transfected DCs as APCs was indicated by the fact that induction of CEA-specific CTLs could be detected routinely following two cycles of stimulation in vitro. This compared

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DCs tran CEA⁺ cell lin were compar ing the fact t⁴ cellular RNA

DCs tran CTLs. Use o⁴ in CTL ass⁴ patients wh⁴ commonly ⁴ nation of th⁴ patients of i⁴ problem is ⁴ CTL assays. from patien⁴

To devel responses th⁴ HLA comp⁴ with IVT ⁴ PBMCs fror⁴ fected with lines (SW14 was measur⁴ as targets. 1⁴ CEA-expre⁴ ic CTL, whi⁴ CEA peptid⁴ of CEA-sp⁴ CEA⁻ KLEI⁴ DCs are effi⁴ measure ⁴ patient's ha

Figure 1. I⁴ response ir⁴ RNA. PBM⁴ vaccinated⁴ DCs pulsed⁴ IVT CEA RI⁴ cells (-▲-⁴ Induction o⁴ specific CT⁴ two stimul⁴ (A) CEA o⁴ experimen⁴

favorably with reports describing the successful induction of primary CTL responses following three or more cycles of stimulation with peptide-pulsed or retrovirally transduced DCs[14-22].

DCs transfected with total RNA isolated from SW403 cells, a CEA⁺ cell line derived from a patient with colon adenocarcinoma, were comparable in stimulating CEA-specific CTLs, notwithstanding the fact that the CEA mRNA species represent a minority of the cellular RNA pool used to transfect DCs.

DCs transfected with CEA RNA are targets for CEA-specific CTLs. Use of peptide-pulsed cells or established cell lines as targets in CTL assays limits the ability to measure CTL responses to patients who express specific HLA alleles, in many instances the commonly expressed HLA-A2 allele. This requires prior determination of the patient's haplotype and excludes from analysis many patients of inappropriate HLA alleles. One possible solution to this problem is to use the CEA⁺ tumor cells from patients as targets in CTL assays. The problem with this approach is that tumor cells from patients are difficult to obtain.

To develop a general approach to measuring patients' CTL responses that does not require the prior knowledge of the patient's HLA composition, we tested whether autologous DCs transfected with IVT CEA RNA could serve as targets in CTL responses. PBMCs from HLA-A2 individuals were stimulated with DCs transfected with CEA RNA or with total RNA isolated from CEA⁺ cell lines (SW1463 and SW1417) or a CEA⁻ cell line (KLEB). CTL lysis was measured using peptide-pulsed DCs or RNA-transfected DCs as targets. DCs transfected with IVT CEA RNA or total RNA from CEA-expressing SW1463 and SW1417 cells stimulated CEA-specific CTL, which lysed either DC pulsed with the HLA-A2-restricted CEA peptide or DCs transfected with CEA RNA (Fig. 2). Induction of CEA-specific CTLs by DCs transfected with total RNA from CEA⁻ KLEB cells was insignificant. Thus, CEA RNA-transfected DCs are efficient targets for CEA-specific CTLs and can be used to measure CTL responses without the need to determine the patient's haplotype.

Induction of primary CTL responses using DCs transfected with RNA encoding the E6 or green fluorescent protein (GFP) antigen. To determine whether the ability to stimulate primary CTL responses by RNA-transfected DCs is unique to the CEA antigen, we tested whether E6 or GFP RNA-transfected DCs can stimulate a CTL response in vitro. E6 is a viral antigen derived from the human papillomavirus type 16, which is expressed in 90% of patients with cervical carcinoma[23]. GFP is a naturally fluorescent jellyfish protein used as a reporter gene in transfection studies. DCs transfected with IVT CEA or GFP RNA were capable of inducing CTL, which recognized and lysed only the antigen-specific target (Fig. 3A). Similarly, E6 and GFP RNA-transfected DCs stimulate CTL responses against their cognate targets (Fig. 3B). DC transfected with RNA were used as targets to measure CTL lysis.

Immature, but not mature, DCs can be efficiently transfected with CEA RNA in the absence of lipid. DCs were transfected with RNA complexed to lipids, as we hypothesized that the lipid/RNA complex may protect the RNA from degradation and enhance RNA uptake[15,26] (Figs. 1-3). The DCs used in this study, obtained by culturing PBMCs for 7 days in the presence of granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4), exhibited predominantly an "immature" phenotype[27]. Immature DCs exhibit high levels of macropinocytosis and express the mannose receptor suggesting that immature DCs are capable of taking up large amounts of nonparticulate forms of antigen[7,28]. We therefore tested whether CEA RNA could be taken up by human DCs in the absence of lipid, as measured by the ability of the RNA-transfected DCs to induce a primary CTL response in vitro. DC transfected with CEA RNA in the absence of lipid were effective at stimulating CEA-specific CTLs (determined by lysis of CEA peptide-pulsed T2 cells and CEA⁺, HLA-A2 SW1463 cells), as compared with DCs transfected with the lipid-RNA complex (Fig. 4). The fact that CEA⁺, SW1463 cells can be recognized as targets suggests that RNA-transfected DCs stimulate high avidity CTLs capable of recognizing endogenously expressed antigen.
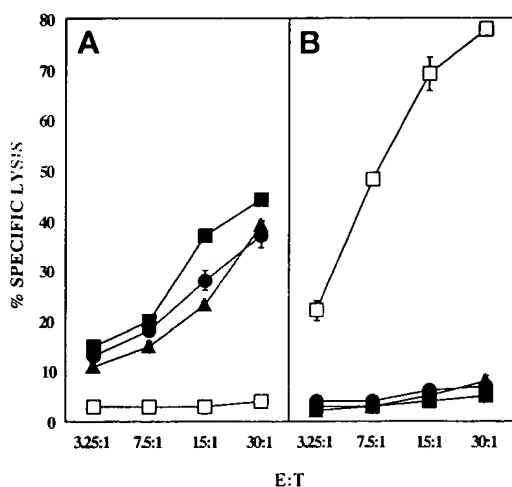
Figure 1. Induction of a primary, CEA-specific CTL response in vitro using DC pulsed with CEA encoding RNA. PBMCs from an HLA-A2, CEA⁻, influenza-vaccinated individual was stimulated with autologous DCs pulsed with M1 peptide (-□-), CEA peptide (-■-), IVT CEA RNA (- -) or total RNA isolated from SW403 cells (-▲-), a CEA-expressing tumor cell line. Induction of M1-specific (memory response) and CEA-specific CTL (primary response) was measured after two stimulations using as targets T2 cells pulsed with (A) CEA or (B) M1 peptides. Data are from four experiments.
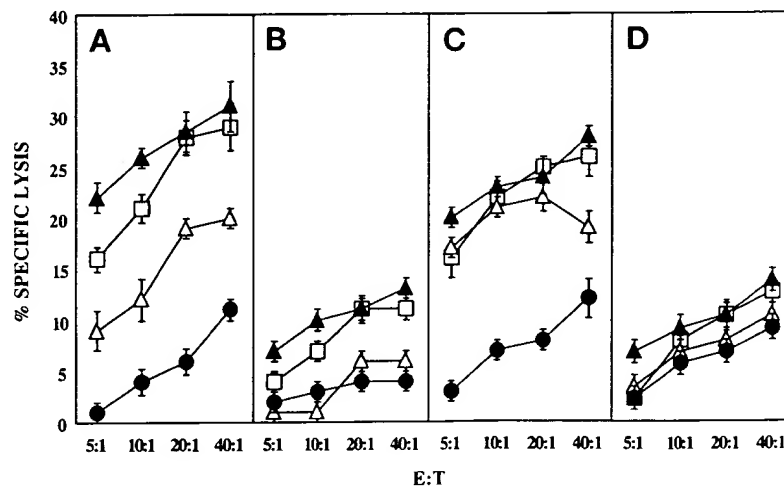
Figure 2. DCs transfected with CEA RNA or pulsed with CEA peptide are targets for CEA-specific CTLs. PBMCs from an HLA-A2, CEA⁻, healthy individual were stimulated with autologous DCs pulsed with IVT CEA RNA (-□-) or with total RNA isolated from SW1463 (-△-) or SW1417 (CEA⁺) tumor cells (-▲-), or KLEB (CEA⁻ tumor cells (-●-). Induction of CEA-specific CTLs was measured using as targets (A) DCs pulsed with CEA peptide or (B) hepatitis C virus peptide (control targets) and (C) DCs transfected with CEA RNA or (D) green flourescent protein RNA (control targets). Data are from four experiments.

To test the hypothesis that uptake of naked RNA is mediated by immature DCs, the PBMC-generated DCs were treated with tumor necrosis factor α (TNFα), before or after CEA RNA transfection. DCs generated in the presence of GM-CSF and IL-4 express intermediate levels of major histocompatibility complex (MHC) class II molecules and B7-1 (CD80) and are mostly CD83^{-} (ref. 29). Following TNFα treatment, PBMC-derived DCs mature, which correlates with the upregulation of class II and CD80 expression, and cells become CD83^{+} (refs. 27,29). Maturation of DCs is also accompanied by an increase in antigen presentation evidenced by enhanced mixed lymphocyte reaction activity of mature versus immature DCs[29]. DCs were generated in the presence of GM-CSF and IL-4, and the large, class II intermediate immature DCs (approximately 50% of the total population) were further purified by cell sorting[14].

Treatment of sorted immature DCs with TNFα caused the upregulation of CD80 and CD83 expression (Fig. 5A–D). Immature DC transfected with CEA RNA and then treated with TNFα were significantly more potent stimulators of CEA-specific CTL than DC treated with TNFα before transfection with RNA (Fig. 5E). Treatment with TNFα had no significant effect on the ability of CEA peptide-pulsed DCs to stimulate a CEA-specific CTL response.

Induction of CD4^{+} T-helper cells by RNA-transfected DC. Efficient induction and persistence of MHC class I-restricted CTL responses in vivo requires the participation of antigen-specific, MHC class II-restricted CD4^{+} T cells[30–33]. Antigen expressed by RNA-transfected DCs will be preferentially channeled into the class I presentation pathway and elicit CD8^{+} CTLs. Thus, vaccination with CEA RNA-transfected DCs will be deficient in generating CD4^{+} T-help that may be necessary for the generation and/or maintenance of an effective CEA-specific CTL response in vivo.

It is possible to redirect endogenously expressed antigens into the class II presentation pathway by appending a leader sequence to the amino end and a lysosomal sorting signal derived from human LAMP-1, to the carboxyl end of the endogenously expressed antigen[34,35]. We have, similarly, created a chimeric CEA cDNA template by using sequences encoding the lysosomal targeting signal of the human LAMP-1 protein to the carboxyl terminal of CEA (CEA-LAMP-1)[34]. CEA RNA and CEA-LAMP-1 RNA were transfected into DCs and used to stimulate PBMCs. To mea-
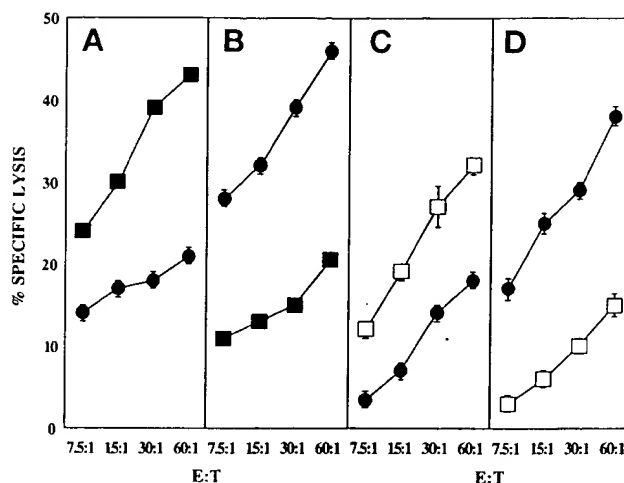
Figure 3. DCs transfected with GFP RNA or E6 RNA stimulate primary, GFP-specific or E6-specific CTLs, respectively. DCs were transfected with IVT CEA RNA (-■-), GFP RNA (-●-) or E6 RNA (-□-) and used as stimulators. DCs transfected with (A) CEA RNA, (B) GFP RNA, (C) E6 RNA, or (D) GFP RNA. Data are from three experiments.
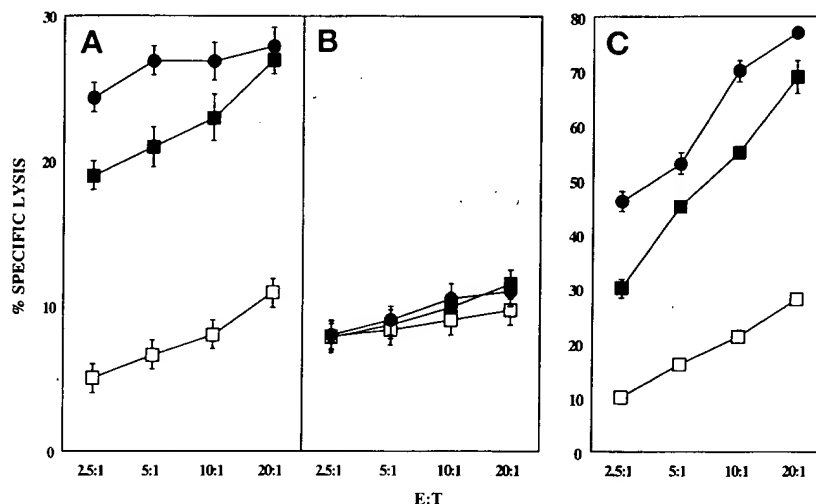
Figure 4. CEA-specific CTL induction by DCs transfected with CEA RNA in the presence or absence of lipid. DCs were transfected with 5 μg IVT CEA RNA (-■-) or OVA RNA (-□-) complexed to 10 μg of DMRIE. Alternatively, cells were transfected with 20 μg CEA RNA in the absence of lipid (-●-) . T2 cells pulsed with (A) CEA peptide or (B) an irrelevant (HCV) peptide and (C) tumor cells expressing CEA (SW1463) were used as targets. Data are from two experiments.
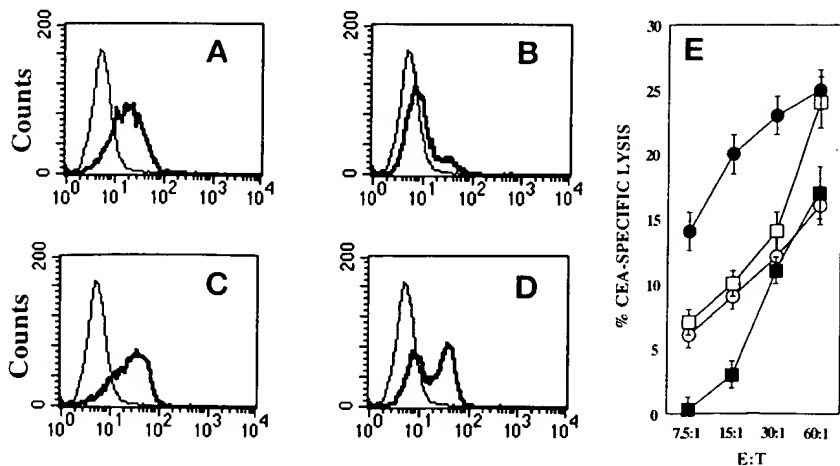
Figure 5. Effect of TNFα treatment on the ability of CEA RNA-transfected DCs to stimulate CTLs in vitro. DCs were generated from PBMCs[29]. The large, class II intermediate cells were purified by cell sorting and analyzed pre- and posttreatment with TNFα (100 ng/ml for 18 h). Pretreatment cells were analyzed for the expression of (A) CD80 (B7-1) and (B) CD83. Cells following treatment were similarly analyzed for (C) CD80 (B7-1) and (D) CD83. (E) Sorted DCs were either incubated with naked CEA RNA (- -) or with CEA peptide (-○-) followed by culture in the presence of TNFα or cultured with TNFα followed by RNA transfection (-■-) or peptide pulsing (-□-). DCs pulsed with CEA peptide and DCs pulsed with HCV peptide were used as targets. Data represent the differences in the lysis of DC+CEA peptide and DC+HCV peptide. The lysis of the control targets was less than 25%. Data are from three experiments.

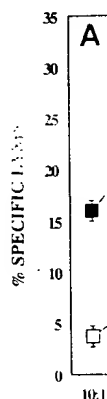sure the induction of CEA-specific T-cell responses, CD4⁺ and CD8⁺ T cells were isolated after the first cycle of stimulation, restimulated with the RNA-transfected DCs and tested for lytic activity against CEA RNA-transfected DC targets. Although activation of CD4⁺ T cells is generally measured using proliferation assays, activated CD4⁺ T cells can also exhibit lytic activity against their cognate tar-

gets in standard cytotoxicity assays[...]. Induction of CTLs, fro purified CD8⁺ T cells in vitro requires the addition of exogenous I 2, which was shown to substitute for activated CD4⁺ T cells in tl stimulation reaction[...]. Consequently, in order to observe the impa of CEA-specific CD4⁺ T cells on CTL induction, stimulation of tl responders was carried out in the presence or absence of IL-2.

In the absence of IL-2, CEA-LAMP-1 RN but not CEA RNA, transfected DCs stimulated robust CEA-specific CD4⁺ T-cell response (Fi 6B), consistent with the hypothesis that tl LAMP sequence has redirected some of the CE product into the class II processing pathwa When stimulation was carried out in the pre ence of IL-2, CEA RNA-transfected DCs we also capable of stimulating CD4⁺ T cells, albu less efficiently than CEA-LAMP-1 RNA-tran fected DCs (Fig. 6A). Endogenously express(antigens that have access to the endoplasm reticulum can also gain access, to a limit(extent, to the class II presentation pathway[...]. It therefore conceivable that the ability of tl unmodified CEA product to elicit a weak, IL-. dependent, CD4⁺ T-cell response is a reflection the fact that it is a secreted product. This al: explains why CEA-transfected DCs can serve . targets for CEA-specific CD4⁺ T cells.

CEA-LAMP-1 RNA-transfected DCs stimu late a more potent CD8⁺ CTL response con pared with CEA RNA-transfected DCs (Fig. 6C which was more apparent when stimulation w: carried out in the absence of IL-2 (Fig. 6D). Th is consistent with previous studies showing th: activation of CD4⁺ T cells helps the induction (CD8⁺ CTL in vitro, especially in the absence (IL-2 (ref. 38). It is likely that the experimen shown in Figure 6D underestimated the magni tude of activity of T-helper cells for CEA CT induction because a portion of the CEA-LAMF 1 product was redirected to the class II presenta tion pathway.
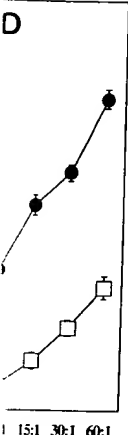
DCs generated from a patient with metastat ic cancer transfected with CEA RNA stimulat CEA-specific CTLs. In the experiment described thusfar, CEA-specific CTLs were stim ulated from PBMCs obtained from healthy vol unteers. We then wanted to determine whethe autologous DCs generated from patients witl advanced metastatic cancer were capable of stim ulating CEA-specific CTLs. DCs were generatec in serum-free AIM-V media as described b! Morse et al.[...] and transfected with naked IV1 CEA RNA. CEA RNA-transfected DCs generatec in serum-free conditions from a healthy individ ual were capable of inducing a potent CEA-spe cific CTL response (Fig. 7A). In this experiment CEA peptide-pulsed DCs were considerably les: effective in stimulating a CTL response (compart with Fig. 1). This difference was seen using botl RNA-transfected DC targets as well as peptide pulsed DC targets (Fig. 7A). DCs transfectec with naked IVT CEA RNA, generated in serum free conditions from a patient with CEA⁺ stag(IIIB adenocarcinoma of the lung, stimulated ; CEA-specific CTL response in vitro (Fig. 7B). This patient was not eligible to participate in an
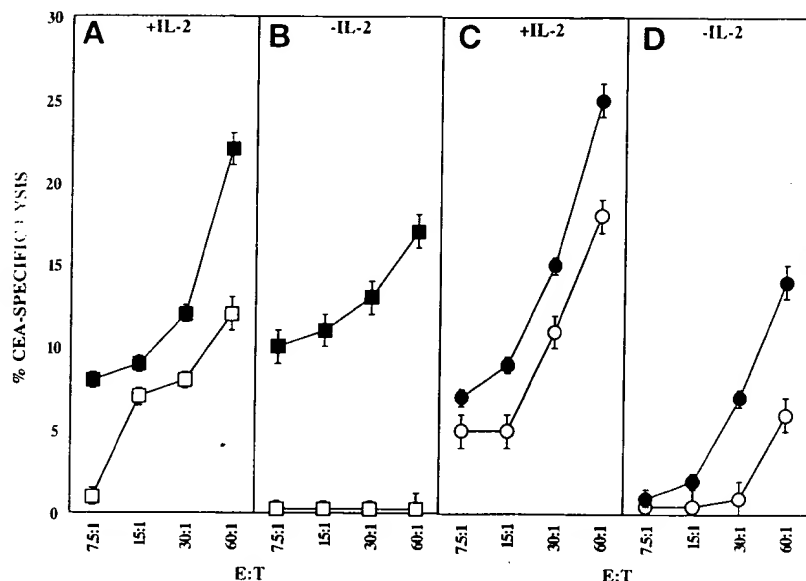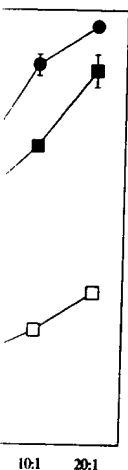

Figure 6. Stimulation of CD4⁺ and CD8⁺ T cells by DCs transfected with chimeric CEA-LAMP-1 RNA. DCs were transfected with CEA RNA or CEA-LAMP-1 RNA complexed to lipid. After the first round of stimulation, CD4⁺ and CD8⁺ T cells were isolated using Dynabeads M-450 CD4 and Dynabeads M-450 CD8 followed by DETACHaBEAD CD4/CD8 to obtain purified, phenotypically and functionally unaltered cells (Dynal, Lake Success, NY). The purity of CD8⁺ and CD4⁺ T cells obtained using Dynabeads was routinely 95% or more by FACS analysis. Purified CD4⁺ and CD8⁺ T cells were restimulated with RNA-transfected DCs for 5 days and used in a CTL assay with DCs transfected with CEA RNA or GFP RNA as targets. Data represent the differences in the lysis of DC+CEA RNA and DC+GFP RNA. The lysis of DC+GFP RNA was 15% or less. (A,B) CD4⁺ T cells as responders. (C,D) CD8⁺ T cells as responders. (A,C) In vitro stimulations in the presence of IL-2. (B,D) In vitro stimulations in the absence of IL-2. Stimulators are either DCs transfected with CEA RNA (-□-; -O-) or DCs transfected with CEA-LAMP-1 RNA (-■-; -●-) Data are from two experiments.
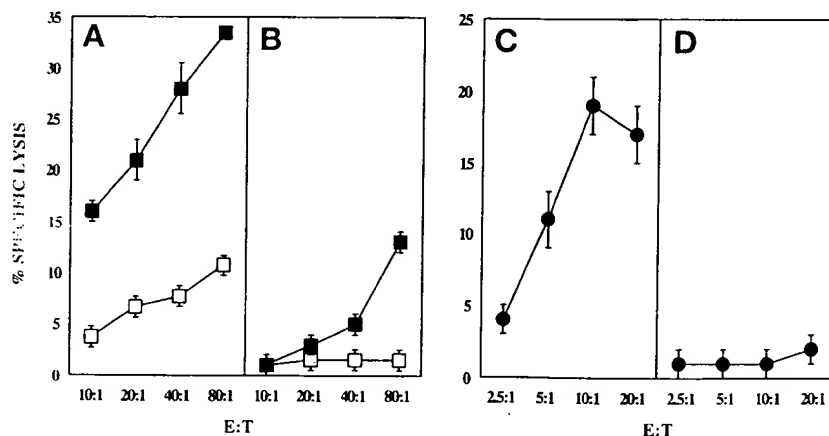
Fι...re 7. DCs generated from a cancer patient used to stimulate CEA-specific CTL re...ponses in vitro. DCs were generated in serum-free AIM V in the presence of GM-CSF and IL-4. DCs were incubated with CEA peptide (-□-) or CEA RNA (-■-) in the absence of lipid. DCs transfected with (A) CEA RNA or (B) GFP RNA were used to measure CEA-specific lysis. (C,D) DCs generated from a patient with metastatic cancer were transfected with naked CEA RNA (-●-) and frozen. Thawed, pulsed DCs were tested as stimulators using autologous, thawed PBMCs as responders. DCs transfected with (C) CEA RNA or (D) GFP RNA were used to measure CEA-specific lysis.

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ongoing clinical trial with CEA peptide-pulsed DC because he was not HLA-A2. Using a similar protocol, we were able to induce in vitro, primary, CEA-specific CTLs from five of eight non-HLA-A2 patients with CEA advanced metastatic cancers (data not shown). Thus, functional DCs can be generated from cancer patients, and stimulation as well as detection of CTLs by RNA encoded antigens is not limited to specific haplotypes.

Discussion

We demonstrated that human DCs transfected with RNA encoded antigen are potent APCs. PBMCs, stimulated by autologous DCs transfected with either IVT CEA RNA or with total cellular RNA isolated from CEA expressing cells, stimulated a primary CEA-specific CTL response in vitro as effectively, if not more effectively, than peptide-pulsed DCs (Figs. 1, 5, and 7). The ability to stimulate a potent CTL response in vitro was not unique to CEA; potent CTL responses were generated with GFP RNA or E6 RNA-transfected DCs (Fig. 3). DCs transfected with naked RNA stimulated potent CTL responses in vitro (Figs. 4, 5, and 7). This would significantly simplify clinical protocols of using RNA-transfected DC-based vaccines. Appending a lysosomal targeting signal to the CEA RNA enhances the induction of CEA-specific CD4 T cells (Fig. 6), providing a strategy to induce T-help that may be necessary to generate and maintain an optimal CD8 CTL response in vivo[30-33].

Vaccination with RNA-transfected DCs, unlike vaccination with peptide-pulsed DCs, is not limited to, and does not require prior knowledge of, the patient's haplotype. CEA-specific CTLs could be generated readily in HLA-A2 (Figs. 1–6) and non-HLA-A2 individuals (Fig. 7). The fact that RNA-transfected DCs are sensitive targets in CTL assays (Figs. 2, 3, and 7) also offers a general method for monitoring induction of CTL in vaccinated patients. Thus far, CEA-specific CTLs were generated and detected with CEA RNA-transfected DCs in five of eight non-HLA-A2, CEA advanced cancer patients, confirming that the CEA product expressed from RNA encoded CTL epitopes for other HLA alleles (Fig. 7).

As it was possible to stimulate in vitro CEA-specific CTL responses with RNA isolated from CEA-expressing cells (Figs. 1 and 2), it is conceivable that DCs transfected with tumor RNA will stimulate CTL responses against other, yet unidentified, tumor antigens. Thus, vaccination with total tumor RNA-transfected DCs could provide a strategy to induce tumor immunity in patients against a broad repertoire of tumor antigens.

One potential drawback, however, of immunizing with unfractionated tumor material as compared with the use of defined tumor antigens, is the increased risk of inducing autoimmune responses with pathological consequences. While no evidence of autoimmunity was seen in animal vaccination studies using unfractionated tumor material[14,15] as a source of tumor antigens, the development of increasingly potent vaccines may very well lead to some autoimmune manifestations.

Experimental protocol

Cell lines. T2 cells[34] (TAP-deficient, HLA-A2, hybrids of T and B lymphoblastoid cells), SW403 (CEA, colon adenocarcinoma, HLA-A2, A3), SW1417 (CEA, colon adenocarcinoma, HLA-A3), KLEB (CEA, HLA-A2 cell line), and SW1463 (CEA, HLA-A2 rectum adenocarcinoma) were used. Cells were maintained in DMEM-F12 supplemented with 10% FCS, 25 mM HEPES, 2 mM L-glutamine, 1 µg/ml insulin, and 1 mM sodium pyruvate.

Precursor-derived DCs. DCs were generated from PBMCs as described by Romani et al.[11] with minor modifications[35]. Adherent PBMCs were cultured in RPMI/10% FCS with 800 U/ml GM-CSF and 500 U/ml IL-4. After 7 days the DCs were harvested and pulsed with antigen. DC were cryopreserved in 90% FCS plus 10% DMSO at 5×10^6/ml and PBMCs to be used responders were cryopreserved at 5×10^7/ml.

The following monoclonal antibodies were used for immunofluorescence staining: anti-CD4, anti-CD14, anti-CD80, anti-CD86, anti-HLA-A,B,C, and anti-HLA-DR (Becton Dickinson, San Jose, CA), and anti-CD83 (Immunotech, Westbrook, ME).

Peptides. The following HLA-A2-restricted peptides were used: influenza virus peptide (M1, amino acids [aa] 58–66: GILGFVFTL); CEA peptide (CAP-1, aa 571–579: YLSGANLNL[16]); hepatitis C virus (HCV) peptide (NS3 protein, aa 1077–1085: CINGVCWTV). Peptides were purchased with free amino and carboxyl ends (Research Genetics, Birmingham, AL), dissolved in serum-free IMDM and stored at -20°C.

Production of IVT CEA RNA and CEA-LAMP-1 RNA. For production of CEA RNA, CEA cDNA in pGEM3Zf+ plasmid was linearized with HindIII for use as templates for in vitro transcription. RNA was made and polyadenylated as described in Boczkowski et al.[15] For cloning of pGEM3Z/CEA-LAMP-1, the EcoRI-Rcal fragment from pGEM3Zf(+)/CEA, which contains the first 2070 bp of the CEA coding region was isolated and ligated into pGEM3Z (Promega, Madison, WI) with oligonucleotides corresponding to the sequence from the Rcal site to the end of the CEA coding region and oligonucleotides coding for the transmembrane region, cytoplasmic tail, and stop codon of the human LAMP-1 protein (Oligos, Etc., Wilsonville, OR). For in vitro transcription reactions the plasmid was linearized by digestion with HindIII.

Isolation of total cellular RNA. Total RNA was isolated from tissue culture cells as described in Boczkowski et al.[15]

Transfecting DCs with RNA. RNA, in 250 µl Opti-MEM (GIBCO, Grand Island, NY) and the lipid DMRIE (Vical, San Diego, CA), in 250 µl Opti-MEM, were mixed in polystyrene tubes at room temperature for 5–10 min[13,15]. The amount of IVT RNA used was 1 µg and the amount of total RNA used was 25 µg, unless otherwise stated. The RNA to lipid ratio was 1:4. The complex was added to DCs (1×10^6 cells/ml) in Opti-MEM and incubated at 37°C for 30 min. DCs were washed and used as stimulators. Alternatively, DCs were pulsed with RNA without DMRIE for 4 h at 37°C.

Induction of primary CTL responses in vitro. Stimulation of PBMCs and expansion of CTLs were as described by Wong et al.[35] Briefly, autologous PBMCs were cocultured with antigen-pulsed DCs at responder:stimulator (R/S) ratio of 10:1 with IL-7 and IL-2. CD8 T cells were harvested on day 12 using Applied Immuno Sciences CD8 microCELLector flasks. The purity of CD8 T cells was routinely ≥90% by fluorescence-activated cell sorting analysis. Two days postpurification T cell blasts were restimulated with antigen-pulsed DCs. CTL assays were done 5 days postrestimulation. Experiments using purified populations of DCs (sorted for large) were done at R/S ratio of 25:1. A standard europium release CTL assay was performed[36]. A standard europium release CTL assay was performed[37]. Europium release was measured by time-resolved fluorescence (Delta fluorometer; Wallac, Gaithersburg, MD). Specific cytotoxic activity was determined with the formula: % specific release = [(experimental release – spontaneous release)/(total release – spontaneous release)] × 100. For Figures 5 and 6 the data is represented as % CEA-specific lysis, which is the difference between the lysis of a CEA-expressing target and a control antigen-expressing target cell. Spontaneous release of the target cells was less than 25%. Standard errors of the means of triplicate cultures was less than 5%.

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   **Perspective: How to make microarray, serial analysis of gene expression,
and proteomic relevant to day-to-day endocrine problems and physiological
systems**
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MEDICAL DESCRIPTORS:
gene mapping; medical genetics; gene function; protein expression; DNA
sequence; *differential* *display*; *dendritic* *cell*; antigen presenting
cell; immunology; neuroimmunology; *cancer* research; two dimensional gel
electrophoresis; matrix assisted laser desorption ionization time of flight
mass spectrometry; mass spectrometry; protein purification; gene
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S1   5   (DENDRITIC (W) CELL?) (S) ((MULTIPLE (W) ANTIGENS) OR (ARR-
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S3   37   (DENDRITIC (W) (CELL OR CELLS)) AND (DIFFERENTIAL (W) (DIS-
                PLAY OR SCREEN OR SCREENING))
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S5   1   RD (unique items)
S6   6   S3 AND (IMMUNOTHERAPY OR CANCER OR TUMOR)
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Perspective: How to Make Microarray, Serial Analysis of Gene Expression, and Proteomic Relevant to Day-to-Day Endocrine Problems and Physiological Systems

Introduction

The recent mapping of the human genome has opened the way to novel technologies for identifying new genes or clustering regulated genes and proteins in a tissue- and cell-specific manner. Microarray and serial analysis of gene expression (SAGE) are quite powerful in this regard, and they generate on a day-to-day basis data banks of gene profile from cells or tissues of animals challenged with different compounds including hormones. Here we review these techniques with their strengths and limits in physiological systems with a particular emphasis on the endocrine system. We also present new developing techniques in proteomic, especially for the analysis of functional proteins and protein-protein interaction. Although we are still at the embryonic stage of the proteomic area, there is no doubt that the current ongoing work will have a great impact in endocrinology. There are also serious drawbacks that have to be taken into consideration to prevent generating data that may either be nonphysiological or difficult to interpret, the most critical one being the experimental design.

Since the crystallization of the first hormone—adrenalin—by Takamine and Aldrich at the beginning of the twentieth century, modern endocrinology kept growing to comprise various fields of research, namely cytology, cellular biology, and more recently molecular biology and genetic. During the past two decades, large-scale sequencing efforts including the Human Genome Project (1) generated initial large-scale databases, and numerous new genes were discovered, some of them encoding proteins with a function still remaining to be unraveled. Recent progress in biotechnology, more particularly in gene expression microarray and SAGE technologies gave us new tools for identifying gene functions and much more (see Table 1). Actually, microarray and SAGE experiments allow us to test the expression of thousands of genes simultaneously and to identify automatically the genes of interest. In the same way, based on the last developments in the technologies of protein separation, quantification, and identification, protein expression profiles are now available with proteomics. Because proteins are final posttranslational products from mRNA, proteomics will give us access to a new database with particular biological significance. Today, in the scientific literature, the number and diversity of data generated from microarray experiments are impressive and there are already numerous reports covering the whole biomedical community. In the field of endocrinology, analysis of both gene and protein expression will be

quite powerful tools to study the regulation of physiological mechanisms triggered or inhibited by hormones. Here we review how to make DNA microarrays, SAGE, and proteomics relevant in modern endocrinology.

DNA microarrays

DNA microarrays have been developed to exploit the huge amount of sequence data generated by large-scale sequencing programs. Briefly, fluorescent probes prepared from the mRNAs of the samples are hybridized onto high-density matrix of thousands or tens of thousands of ordered DNA known sequences representing specific genes. Hybridization intensity is determined for each represented gene on the matrix, allowing the quantitative comparison of the expression levels of almost all transcripted genes in two or more RNA samples. Two different microarray technologies are available; the oligo microarrays (e.g. from Affymetrix, Inc., Santa Clara, CA) and the cDNA microarrays that differ with the length of DNA sequences (from 25 oligomers to several hundred oligomers, respectively) synthesized or grafted on the matrix, the type of the matrix (glass, nylon, membranes, and other formats) and, finally, the data processing. Arrays are customizable in DNA species and in number of genes represented. When using two different samples (treated and control), we can compare the gene expression profiles between them and then determinate how the cell or tissue regulates its genes in a specific environment. DNA microarrays are like powerful automatic RNA differential display experiments, without the need to both sequence and quantify the bands of interest. Moreover, cDNA microarray sensitivity allows working with as few as 10 μg RNA, for instance about 100,000 cells (2), which is compliant with the small quantities of clinical samples needed in endocrinology. Thus, DNA microarrays are suitable tools for endocrinology studies, such as the analysis of the cellular response to a specific stimulus. For example, Feng et al. (3) identified from mouse livers 45 genes not previously identified as thyroid hormone-responsive genes. In another example, Dupont et al. (4) have used cDNA microarray technology to define the specificity of insulin vs. IGF-1 signaling. Of the 2221 genes tested on cDNA microarrays, 30 genes significantly increased in presence of IGF-1 but not by insulin, and 27 of them were not previously reported as being IGF-1-responsive genes. Work done in other fields can also be quite powerful to unravel genes associated with the endocrine system not necessarily expected to be regulated or even expressed in a particular group of cells. In exploring how dendritic cells modulate the immune system in response to different pathogens, Huang et al. (5) found that activinβa is one of the highly up-regulated genes when antigen-presenting cells are exposed to Esche-

TABLE 1. List of various studies in the field of endocrinology that exploited DNA microarray or SAGE technologies

Study	Tissue	Author	DNA microarray, number of genes screened	SAGE, number of tags screened
Thyroid hormone	Thyrotropic tumor	Wood et al. (53)	1176	
E2, experimental autoimmune encephalomyelitis	Transgenic mouse	Matejuk et al. (54)	12000	
CpG DNA hormone-receptor status	Breast tumor tissues	Yan et al. (55)	7776	
Progesterone receptors	Human breast cancer cells	Richer et al. (56)	588	
Insulin and IGF1	Mouse fibroblast NIH-3T3	Dupont et al. (4)	2221	
GnRH	Mouse LβT2 gonadotrope cells	Wurmbach et al. (57)	956	
GH	Rat neonatal ventricular myocytes	Lu et al. (58)	9223	
Sterol regulatory element-binding protein-1, insulin receptor	Insulin receptor substrate 2 (−/−) mouse liver	Tobe et al. (59)	11000	
ERα	Breast carcinoma cells	Gruvberger et al. (60)	6728	
PRL, GH, ACTH	Pituitary adenomas	Evans et al. (61)	7075	
Hypophysectomy	Rat heart, liver, and kidney	Flores-Morales et al. (62)	3000	
Thyroid hormone	Hypothyroid or T$_3$-treated mice liver	Feng et al. (3)	2225	
Hormone refractory prostate cancer	CWR22 and CWR22R prostate cancer xenografts	Bubendorf et al. (63)	5184	
Corticosteroid	Rat hippocampus	Datson et al. (31)		≈20000
Androgen	Prostate cancer cells	Xu et al. (33)		83489
Aldosterone vasopressin	Kidney cells	Robert-Nicoud et al. (30)		169721
Estrogen	Human breast cancer cells	Inadera et al. (64)		≈30000
Atherogenic stimulus	Human arterial endothelial cells	De Waard et al. (22)		12000

richia coli. It will not be long before we clarify the physiological relevance of such regulation and the presence of this hormone in the innate immune system. One may expect unraveling very fine and unexpected mechanisms modulated by activin in the innate immunity, which is the case for SMAD3 that is one of the intracellular mediators of the activin receptors (6). ·

As a major application of DNA microarray, expression arrays can be used to understand multigenic diseases such as many cancers (7, 8). "Fold difference," *e.g.* the ratio of gene expression in a treated sample over the control sample is used as quantitative measurements of the differential expression, to generate a clustering of genes. These clusters can be arranged hierarchically or spatially to form self-organized maps (9, 10). Expression cluster can be used to search common motifs of genetic regulation to find new regulatory mechanisms. Another application of DNA microarrays is the finding of new functions of genes by association of gene expression. Bioinformatics, with the use of algorithms, provided tools to trace out metabolic pathways, cellular interactions and to discern genetic networks (11–13). However, before using these predicting algorithms, it is imperative to distinguish between significant fold difference values and false-positive results to avoid reporting data that may actually not be physiologically relevant (14). Replicates are also required to lower the experimental noise and to display low level of differential expression significantly. For these reasons, it is imperative to confirm the presence of newly discovered expressed genes in a specific tissue by Northern blot, real time RT-PCR or *in situ* hybridization. The latter being the best approach, because it permits not only to validate, but also visualize the expression pattern and even the type(s) of cells expressing the transcript during a specific time, treatment, or changes in plasma hormone levels.

As described above, DNA microarrays are useful to identify genes that are markers of multigenic diseases. When these markers will be well defined, the design of DNA microarrays could then be customized to test simultaneously all these markers for a diagnostic use. Furthermore, DNA microarrays will be very efficient tools to detect the response to therapy, such as the prostate tumor response to androgen withdrawal, and to plan more appropriated medical treatments. In this regard, Bubendorf *et al.* (15, 16) have described another use of microarrays, not as DNA microarrays, but as tissue microarrays. Concisely, hundreds or thousands of 0.6-mm diameter tissue cylinders are arrayed on a glass slide allowing the instantaneous analysis of every sample with either immunohistochemistry, fluorescence *in situ* hybridization or RNA *in situ* hybridization. Therefore, these tissue microarrays could be quite useful for clinical studies, such as paired analysis of prostate cancer biopsies.

When DNA microarrays are used in a whole-genome expression analysis, large volumes of data are generated raising computational requirements (17). Many microarray data are now available on public on-line databases (*e.g.* Stanford Microarray Database; http://genome-www5.Stanford.EDU/MicroArray/SMD/). However, analysis of DNA microarray data are limited to relative comparison between samples. Furthermore, oligo microarray raw original data have to be processed for bias corrections like multiplicative effects (*e.g.* dif-

ference in the total mRNA concentration of samples), additive effects (*e.g.* background), position effects on the microarray and nonlinear effects (saturation of detectors of the hybridization intensity). All these biases emphasize the need to have access to complete raw data sets to provide a significant comparison of array results when processing data with normalization curves. Moreover, the identification number of each gene on the array is usually different from one microarray brand to another one, requiring the usage of Unigene nomenclature to share and compare different platform microarray data (18, 19). Expression profiling of large amount of clinical samples is very efficient with microarrays, although SAGE is more suitable than microarrays for identifying new genes or RNA that are alternatively spliced, because microarrays allow only to test known genes on the chip.

SAGE

SAGE is a method based on the isolation of unique short sequence tags from individual polyadenylated RNAs and on concatenation of these tags serially to facilitate their sequencing, and therefore examine gene expression profiling (20). Polyadenylated RNAs are captured from cell lysates with oligo-deoxythymidine-coated beads and are reversed transcripted in cDNA. Isolation of tags from cDNA is performed with the formation of unique 5' end position within the 3' end part of each cDNA by cleavage with anchoring enzyme. Tags are released using different strategies (21) and are concatemerized into long DNA sequence. Finally, concatemer clones are sequenced and tag sequences are BLAST against GenBank to allocate a gene identity to each tag. Basic tag counting permits the determination of absolute tag abundance.

There are several limitations to keep in mind when using SAGE. For example, some transcripts could lack an anchoring enzyme site and would not be tagged. There is also an inherent low sequencing error rate that alters the accuracy of the tag count and increase mistrust of the abundance of tags with low count. Another problem is the making of valid tag to gene assignments while the large majority of transcript source sequences available in GenBank are expressed sequenced tag sequences. These are usually only single-pass sequenced, making possible to contain sequence errors. Additionally, tags are very short sequences (usually 9–11 bp), and two genes can share the same tag. A further source of the problem is when making a tag-to-gene assignment for a tag without corresponding entries in databases. Because the sequence available in the 11-bp tag is extremely limited, the cloning of the full-length genes then becomes difficult.

On the other hand, SAGE strengths are remarkable. First of all, SAGE data represent absolute RNA expression levels that are easily portable and directly comparable to existing SAGE database. Actually, more than three million transcript tags are already available on the Internet (http://bioinfo. amc.uva.nl/HTM-bin/index.cgi/; http://www.sagenet.org; http://www-dsv.cea.fr/thema/get/sade.html; http://www.ncbi.nlm.nih.gov/SAGE; http://www.urmc.rochester.edu/smd/crc/swindex.html; http://genome-www4.stanford.edu/cgi-bin/SGD/SAGE/querySAGE), and the number of libraries keeps growing. SAGE also allows the potential identi-

fication of new transcripts that are not already recorded in GenBank.

SAGE is compliant to analyze the differential gene expression between diseased and normal tissues, and studies have been reported on diseases, such as arteriosclerosis (22) and human immunodeficiency virus infection (23). This technology has recently been used to identify the full set of genes expressed by mammalian rods that provided evidence that half of all cloned human retinal disease genes are selectively expressed in rod photoreceptors (24). SAGE has been widely used in the fields of immunology and neuroimmunology as well as oncology (25–28). For example, Polyak *et al.* (29) reviewed some applications of SAGE in cancer research and described more particularly the analysis of specific gene expression patterns in cancer cells and also the identification of regulatory targets of oncogenes and tumor suppressor genes. Some interesting applications of SAGE have also been reported in endocrinology, such as the changes in the transcriptome of kidney cortical collecting duct principal cell line induced by aldosterone and vasopressin (30). After sequencing approximately 170,000 transcript tags, roughly 15,000 tags were assigned to identified genes, whereas 3,642 tags failed to match with known mouse sequences. This work revealed 34 aldosterone-induced transcripts, 29 aldosterone-repressed transcripts, 48 vasopressin-induced transcripts, and 11 vasopressin-repressed transcripts, some of them having been validated by Northern blot hybridization or real-time RT-PCR (30). With a similar strategy, Datson *et al.* (31) reported the identification of over 200 putative corticosteroid-responsive genes in rat hippocampus that are regulated via mineralocorticosteroid and glucocorticosteroid receptors. These corticosteroid-responsive genes could provide new insights on the role of glucocorticoids in the brain and their potential involvement in the mechanisms leading to neuroprotection and/or neurodegeneration (for a review, see Ref. 32). Another example of SAGE application in endocrinology is the exposure of cancer prostate LNCaP cells to synthetic androgen, which resulted in 136 induced genes and 215 repressed genes when compared with untreated control cells (33). Most of these androgen-regulated genes were not previously described, underlying again the role of SAGE technology to discover new genes and their functions.

Proteomic

Although a good correlation between transcript and protein expression levels is expected, mismatches can occur (34, 35), because posttranscriptional mechanisms control the turnover and the posttranslational modifications of proteins. Moreover, alternative splicing can generate multiple transcripts that enhance the diversity of protein functions. Thus, information about protein expression is both important and complementary to genomics, opening therefore the way to the proteomic area that is clearly under way at this time.

Like genomics, proteomics take advantage of the later developments in high technology to allow, as initial goal, the mapping of the proteome of biological systems. One primary tool in proteomics is the protein separation by two-dimension gel electrophoresis (2DGE) (36) followed by immunoblotting or protein visualization with either a staining (Silver,

Coomassie, or SYPRO Ruby) or other chemoluminescence or radiolabeling methods. 2DGE techniques provide the first protein fingerprints in a single picture proteins extracted from tissues or cells. Comparative picture analysis with computers then leads to the identification of differentially expressed proteins guiding their extraction from gel. One limitation of 2DGE fingerprints is the difficulty to compare and quantify low protein expression levels, but this problem could be bypassed using isotope-coded affinity tags (37).

Until recently, proteins were mainly sequenced by Edman method, which was limited in sensitivity and restricted in N-terminal modifications. Actually, mass spectrometry (MS) seems the method of choice to characterize proteins (reviewed in Refs. 38 and 39). After digesting of extracted proteins with trypsin, peptide masses are commonly measured using either matrix-assisted laser desorption ionization—time of fly (MALDI-TOF) MS or tandem MS and are compared with protein digest databases or nucleic acid databases to characterize the sample. Numerous mass spectrometric databases are already available via Internet. 2DGE associated with MALDI-TOF MS has been used in various studies to achieve protein expression differential display (40, 41) but not yet in the field of endocrinology (at least at time that this review was written). It will, however, not be long before seeing reports using these powerful approaches to determine the role of hormones on protein profile in tissues and cells in culture.

In addition to these descriptive proteomic approaches, there are other tools that are currently under development to answer the new challenges of functional proteomics. Like cDNA microarrays, spotted arrays based methods have been developed for high-throughput screening of protein-protein interactions or protein-small molecule interactions. Actually, there are already proof of principles for protein or peptide microarrays (42, 43) and antibody arrays (44). Moreover, Cyphergen Biosystems have developed the ProteinChip System, which consists on capturing specifically proteins of the sample on a specific matrix presenting antibodies or proteins, and to desorb interacting molecules with a technique called surface-enhanced laser desorption/ionization, and the resulting peptide masses are measured by MS (45). These devices are useful to high-throughput screening and clustering of interacting proteins, although they do not provide information on the changes that occur during protein interactions. Other limitations of protein arrays are both the stability of the grafted proteins and their *in vitro* folding. Furthermore, short peptide microarrays do not take into account the effects of the protein environment. As an alternative to protein microarrays, Ziauddin and Sabatini (46) have developed a promising microarray of cells expressing defined cDNA; cells auto-transfect themselves with the local cDNA when they are cultured on an ordered cDNA array. The microarray of the resulting phenotypes can be rapidly screened for drug targets.

Proteomics encounter some technologic limitations at the level of protein purification (2DGE). To help solve these challenges, microfluidic devices have been developed (47). These miniature devices enclose channels, reservoirs and reaction chambers into two sealed plates, and can be interfaced to a mass spectrometer via an electrospray ionization emitter. Microfluidic devices can be used to digest protein as well as separate and purify proteins with a greater sensitivity and speed than 2DGE. Protein separation by 2DGE and MS analysis can also be coupled with the so-called "molecular scanner" (48). Briefly, this device allows in the same time the digestion and the transfer of protein spot from the 2DGE to a membrane that is scanned using MALDI-TOF MS.

Future directions and concluding remarks

Proteomic tools are clearly limited by the technology, but they are promising and there is no doubt that we will assist to a proteomic revolution in the next few years. Endocrinology will greatly benefit from this revolution that will help finding new hormones and small peptides. It will also be possible to provide functional prediction in combining gene and protein expression data with other data source, such as published literature—via automatic information extraction (49–52)— and DNA and protein sequence database. In modern endocrinology, DNA microarrays and SAGE can indubitably lead us to identify the hormone-responsive transcriptomes, and proteomics will allow the identification of hormone-responsive proteomes (see Fig. 1). DNA microarray and SAGE as well as 2DGE are now standard tools in numerous laboratories. Future progress will certainly come from computational fields, more particularly from algorithm improvement and also from the integration of all biological information databases (texts, genes, proteins, structure) after data standardization. *In silico* exploitation of this huge amount of information is a promising way to analyze powerfully an integrated atlas of both the transcriptome and proteome. Figure 1 illustrates how these approaches can be integrated for providing useful data from the gene to the physiological function.

The cost and the large amount of data that are generated by these new technologies are the current bottlenecks for making them available on a day-to-day basis for endocrinologists. Another crucial point that is frequently forgotten when engaging microarray, SAGE, and proteomic assays is the actual experimental design either *in vivo* or *in vitro*. How physiological is a data bank generated from tissues of animals treated with large doses of dexamethasone, for example, rather than physiological concentrations of glucocorticoids that are secreted during stress? It is obvious that boosting the system will be helpful to identify new genes or clusters of regulated genes and proteins, but whether such phenomena will be occurring during normal endocrine changes remain an open question. The blood may also be a potential problem, especially in highly vascularized tissues and when inflammatory events take place. It is indeed quite difficult to determine whether the group of regulated genes is produced by parenchymal cells within the tissue itself or from blood borne immune cells. This is especially crucial during any types of immune stimuli, but also during normal circumstances where most tissues are filled with blood and its elements. *In situ* hybridization is an important step for validating the data generated via either microarray or SAGE techniques. This approach has numerous advantages, including the pattern of expression and cellular source of the
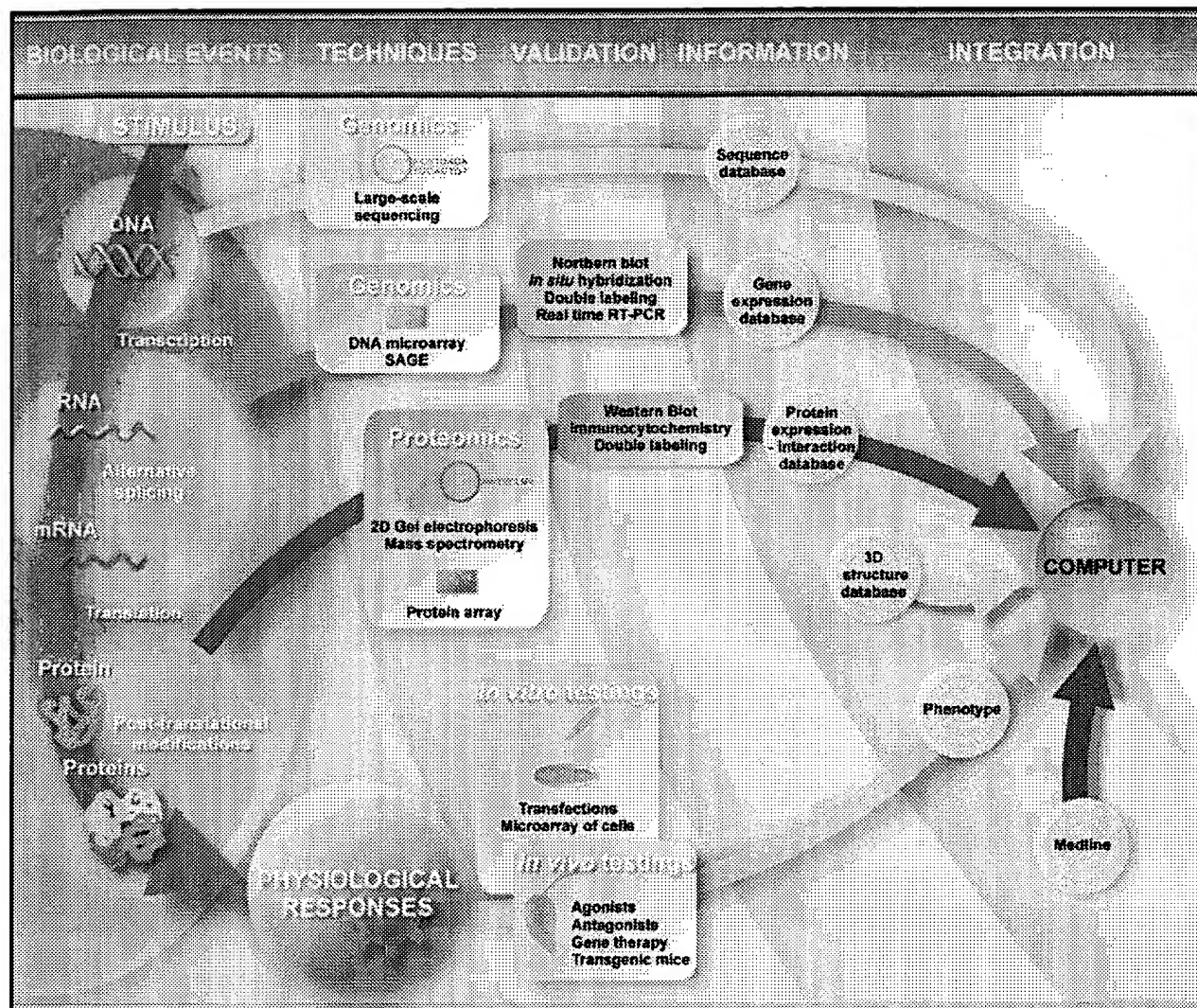
FIG. 1. Schematic illustration depicting DNA microarrays, SAGE and proteomics in a vast network of experimental tools and the resulting information. The different levels of a biological system are represented in relationship with the technology that can be applied, experimental validation of the results and data integration. All the steps are intimately interrelated to provide accurate information in an integrated fashion and of significant physiological relevance.

hybridized genes. Such histological identification is nevertheless best obtained in perfusing and fixing the whole animal with paraformaldehyde. Therefore, a lack of positive hybridization signal may not necessarily invalidate the microarray and SAGE data, because these regulated genes may be expressed by blood irrigating cells that are no longer present in tissues of perfused animals. One can appreciate this concern when gene analysis is performed on tissues, such as injured brains and spinal cords, where a large cluster of regulated genes will most likely be of systemic and not cerebral origin. It is therefore quite important to take these considerations into account to avoid such mismatches and obvious problems in the data interpretation.

The new technologies for gene and protein analysis will be quite helpful for our field of research, but one must always keep in mind that experimental design is the first and most important step. If this is wrong, even the most brilliant genetician and bioinformatician will be useless in analyzing the pile of data that will be generated. On the other hand, small and rigorously well-controlled experiments are likely to generate data of high physiological relevance that will be applicable on a day-to-day basis. Such a large-scale project requests the need of numerous collaborators in almost of all fields of health research, but physiologists will play a determinant role to make sure that all this effort is worthy.

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        S8        0  (DENDRITIC (W) CELLS) (S) (POLYEPITOPES OR POLYEPTITOPE)
?s polyepitopes
        S9        6  POLYEPITOPES
?rd
...completed examining records
     S10        2  RD (unique items)
?t s10/3,k/all


  10/3,K/1      (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
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09381468    21144746    PMID: 11249673
  The polyepitope approach to DNA vaccination.
  Smith S G
  ICRF Cancer Medicine Research Unit, St James's University Hospital,
Beckett Street, Leeds, LS9 7TF, UK. mrpsgs@cancermed.leeds.ac.uk
  Current opinion in molecular therapeutics (England)    Feb 1999,  1   (1)
p10-5,  ISSN 1464-8431   Journal Code: 100891485
  Document type: Journal Article; Review; Review, Tutorial
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed


  ... strategies  and the more recent results that confirm the potential of
DNA encoded polyepitope vaccines. Future directions that may aid the design
of more effective *polyepitopes* will also be discussed.


  10/3,K/2      (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
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09143373    20444234    PMID: 10986397
  Differential  antigenicity  of  recombinant polyepitope-antigens based on
loop- and helix-forming B and T cell epitopes.
  Theisen D M; Bouche F B; El Kasmi K C; von der Ahe I; Ammerlaan W; Demotz
S; Muller C P
  Department  of  Immunology  and  WHO  Collaborating  Center  for Measles,
Laboratoire National de Sante, B.P. 1102, L-1011 Luxembourg, Luxembourg.
  Journal of immunological methods (NETHERLANDS)   Aug 28 2000,  242  (1-2)
  p145-57,  ISSN 0022-1759   Journal Code: 1305440
  Document type: Journal Article
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed


  ... of even sequential BCEs and the processing of TCEs are both sensitive
to  their  molecular  environment  it is difficult to predict the antigenic
properties  of  *polyepitopes*. However, with the permutational approach we
have    developed    several    polyepitope    constructs    [(L(4)T(4))(2),
(L(2)T(2))(4), (H(2)T(2...

... and B cells. Several constructs induced sera that reacted with reporter
peptides,  demonstrating  that  the sequential nature of the viral epitopes
was  conserved  in  the  *polyepitopes* . Although  several sera contained
antibodies  directed  against amino acids critical for neutralization, only
one  construct  induced  antibodies  that cross-reacted with the virus. Our
results...
?ds


Set     Items    Description
S1          5    (DENDRITIC (W) CELL?) (S) ((MULTIPLE (W) ANTIGENS) OR (ARR-
                 AY (W) OF (W) ANTIGENS))
S2          3    RD (unique items)
S3         37    (DENDRITIC (W) (CELL OR CELLS)) AND (DIFFERENTIAL (W) (DIS-
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                  PLAY OR SCREEN OR SCREENING))
S4          3    S3 AND (POLYEPITOPES OR ARRAY OR MULTIPLE)
S5          1    RD (unique items)
S6          6    S3 AND (IMMUNOTHERAPY OR CANCER OR TUMOR)
S7          3    RD (unique items)
S8          0    (DENDRITIC (W) CELLS) (S) (POLYEPITOPES OR POLYEPTITOPE)
S9          6    POLYEPITOPES
S10         2    RD (unique items)
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           37    S3
       114792    IMMUNOTHERAPY
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?s (APC? or (dendritic (w) cell?)) and ((multiple (w) antigens) or (polyeptiopes))
Processing
        32935    APC?
        87270    DENDRITIC
      8632717    CELL?
        44455    DENDRITIC(W)CELL?
      1074138    MULTIPLE
       710382    ANTIGENS
          639    MULTIPLE(W)ANTIGENS
            0    POLYEPTIOPES
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                 ANTIGENS) OR (POLYEPTIOPES))
?rd
...completed examining records
     S13    3    RD (unique items)
?t s13/3,k/all
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13/3,K/1   (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.

09280394   21017579   PMID: 11143904
  Oral immunomodulation therapy in rheumatoid arthritis.
  Meyer O
  Service de rhumatologie, h pital Bichat, Paris, France.
  Joint, bone, spine - revue du rhumatisme (France)   2000, 67   (5)
  p384-92, ISSN 1297-319X   Journal Code: 100938016
  Document type: Journal Article; Review; Review, Tutorial
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed

  ... present them to T cells. Intact antigens can penetrate through
specialized Peyer's patch enterocytes called 'M cells'; they are then
degraded and presented by *dendritic* *cells* to Peyer's patch T cells. The
influx of *multiple* *antigens* through the gastrointestinal mucosa usually
results in tolerance. High-dose tolerance is due to T cell deletion or
anergy, whereas low-dose tolerance involves activation...

  13/3,K/2   (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.

08766970   20048304   PMID: 10581603
  Vaccine therapy for patients with melanoma.
  Haigh P I; Difronzo L A; Gammon G; Morton D L
  Sonya Valley Ghidossi Vaccine Laboratory, John Wayne Cancer Institute,
Saint John's Health Center, Santa Monica, California, USA.
  Oncology (Williston Park, N.Y.) (UNITED STATES)   Nov 1999, 13 (11)
  p1561-74; discussion 1574 passim, ISSN 0890-9091   Journal Code: 8712059
  Document type: Journal Article; Review; Review, Academic
  Languages: ENGLISH
  Main Citation Owner: NLM

...of the lack of effective conventional modalities. The most extensively studied melanoma vaccines in clinical trials are whole-cell preparations or cell lysates that contain *multiple* *antigens* capable of stimulating an immune response. Unfortunately, in the majority of studies, immune responses to these vaccines have not translated into a survival advantage. Advances...

... poorer efficacy due to immunoselection and appearance of antigen-negative clones within the tumor. Novel approaches to vaccine design using gene transfection with cytokines and *dendritic* *cells* are all promising. However, the induction of immune responses does not necessarily confer a therapeutic benefit. Therefore, these elegant newer strategies need to be studied...

13/3,K/3   (Item 1 from file: 73)
DIALOG(R)File   73:EMBASE

12001396   EMBASE No: 2003112743
   Tumor-associated antigens as tools in immunodiagnostics and immunotherapy of breast cancer
   EINSATZ TUMORASSOZIIERTER ANTIGENE IN DER IMMUNTHERAPIE UND
IMMUNDIAGNOSTIK DES MAMMAKARZINOMS
   Guckel B.; Meuer S.; Bastert G.; Wallwiener D.
   Dr. B. Guckel, Frauenkl. E.-Karls-Univ. Tubingen, Calwerstrasse 7, 72076
   Tubingen Germany
   AUTHOR EMAIL: brigitte.gueckel@uni-tuebingen.de

   ...of antigen-defined vaccines. Direct in vivo administration of peptides in combination with adjuvants suitable for establishing effective immune responses or ex vivo loading of *dendritic* *cells* with tumor-specific epitopes are target-specific immunation approaches. Cocktails of synthetic peptide epitopes should permit targeting *multiple* *antigens* while avoiding the development of antigen-loss variants. Most clinical phase I/II trials to date indicate that cellular or peptide-based vaccines are safe...
MEDICAL DESCRIPTORS:
tumor immunology; immunoassay; immune system; antigen presentation; cancer immunology; immunostimulation; tumor cell; cell fusion; hybrid cell; antigen presenting cell; antigenicity; tumor immunity; drug screening; *dendritic* *cell*; human; clinical trial; review
?ds

Set	Items	Description
S1	5	(DENDRITIC (W) CELL?) (S) ((MULTIPLE (W) ANTIGENS) OR (ARR-AY (W) OF (W) ANTIGENS))
S2	3	RD (unique items)
S3	37	(DENDRITIC (W) (CELL OR CELLS)) AND (DIFFERENTIAL (W) (DIS-PLAY OR SCREEN OR SCREENING))
S4	3	S3 AND (POLYEPITOPES OR ARRAY OR MULTIPLE)
S5	1	RD (unique items)
S6	6	S3 AND (IMMUNOTHERAPY OR CANCER OR TUMOR)
S7	3	RD (unique items)
S8	0	(DENDRITIC (W) CELLS) (S) (POLYEPITOPES OR POLYEPTITOPE)
S9	6	POLYEPITOPES
S10	2	RD (unique items)
S11	0	S3 AND (IMMUNOTHERAPY)
S12	5	(APC? OR (DENDRITIC (W) CELL?)) AND ((MULTIPLE (W) ANTIGEN-

Bone Marrow–generated Dendritic Cells Pulsed with Tumor Extracts or Tumor RNA Induce Antitumor Immunity against Central Nervous System Tumors

By David M. Ashley,* Brenda Faiola,[‖§] Smita Nair,[‖] Laura P. Hale,[‡] Darell D. Bigner,[‡] and Eli Gilboa[‖§]

*From the *Department of Pediatrics, [‡]Department of Pathology, [§]Department of Immunology, and [‖]Department of Surgery, Duke University Medical Center, Durham, North Carolina 27710*

Summary

Recent studies have shown that the brain is not a barrier to successful active immunotherapy that uses gene-modified autologous tumor cell vaccines. In this study, we compared the efficacy of two types of vaccines for the treatment of tumors within the central nervous system (CNS): dendritic cell (DC)-based vaccines pulsed with either tumor extract or tumor RNA, and cytokine gene–modified tumor vaccines. Using the B16/F10 murine melanoma (B16) as a model for CNS tumor, we show that vaccination with bone marrow–generated DCs, pulsed with either B16 cell extract or B16 total RNA, can induce specific cytotoxic T lymphocytes against B16 tumor cells. Both types of DC vaccines were able to protect animals from tumors located in the CNS. DC-based vaccines also led to prolonged survival in mice with tumors placed before the initiation of vaccine therapy. The DC-based vaccines were at least as effective, if not more so, as vaccines containing B16 tumor cells in which the granulocytic macrophage colony-stimulating factor gene had been modified. These data support the use of DC-based vaccines for the treatment of patients with CNS tumors.

The concept that the brain is an immunologically privileged site has been supported clinically by the failure of central nervous system (CNS) tumors to respond to immunotherapy protocols that were successful systemically (1, 2). Recently, several groups including ours have described active immunotherapy protocols using intradermal vaccines of genetically modified tumor cells as being effective in rodent brain tumor models (3, 4). However, animal studies have shown that immunization with CNS-derived tumor material can induce fatal experimental allergic encephalitis (5). Due to the limited ability of reliably obtaining and growing a high percentage of tumor specimens without contamination by normal nervous tissue, the application of similar approaches to human patients with brain tumors may therefore carry the risk of causing such autoimmune complications (6, 7).

The dendritic cell (DC) network is a specialized system for presenting antigen to naive or quiescent T cells, and it plays a central role in the induction of T and B cell immunity in vivo. Immunizations using DCs loaded with tumor antigens may, therefore, represent a powerful method of inducing antitumor immunity. Recent studies have shown that immunizing mice with DCs pulsed with specific antigens can prime a CTL response that is tumor-specific and engenders protective tumor immunity in the treated mice (8–10). Immunization using defined tumor antigens is, how-

ever, limited at present to a handful of human tumor types in which candidates for tumor rejection antigens have been identified (11). More recently, effective tumor immunity in mice was induced using DCs pulsed with unfractionated tumor-derived antigens in the form of peptides (12, 13), cell sonicates (14), or messenger RNA (mRNA; 15). The advantages of vaccinating with total tumor-derived material are that the identity of the tumor antigen(s) need not be known and that the presence of multiple tumor antigens reduces the risk of antigen-negative escape mutants. The potential benefit of using total tumor antigens in the form of mRNA is that it can be amplified from a small number of tumor cells. Hence, DC vaccine treatment may be extended to patients with brain tumors from which only a small, possibly microscopic, biopsy can be taken for diagnosis. Furthermore, isolating bona fide tumor cells from patient specimens by ex vivo purification methods and combing this with the use of RNA subtractive hybridization techniques may reduce the concentration of self, potentially autoreactive, antigens in the vaccine preparation. This would be of crucial importance for vaccinations with CNS tumor-derived antigens, as it may diminish the risk of severe autoimmune complications.

The studies presented here evaluated and compared the efficacy of DC-based tumor vaccines pulsed with either tumor extract or tumor-derived total RNA, with that of tu-

mor vaccines in which the gene for GM-CSF had been modified. The vaccines were studied in a model of active immunotherapy for CNS tumors.

Materials and Methods

Tumor Cell Lines and Animal Models. The B16/F10 murine melanoma cell line (B16) derived from a spontaneous melanoma in a C57BL/6 mouse (H-2^b) was provided by I. Fidler (M.D. Anderson Cancer Center, Houston, TX; reference 16). The SMA 560 cell line was derived from an intracerebral transplant of a spontaneous astrocytoma from a VM/Dk mouse (H-2^b) (17). The SMA 560 cell line was chosen as a control for the B16, since both cell lines are derived from neural crest. EL-4 (H-2^b) murine thymoma cells were obtained from American Type Tissue Culture Collection (Rockville, MD). Cell lines were grown in zinc option medium (GIBCO BRL, Gaithersburg, MD) containing 5% (vol/vol) FCS. All cell lines were shown to be free from *Mycoplasma* contamination as previously described (18). All experiments used 6–12-wk-old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), which were maintained in a virus-free environment in accordance with the Laboratory Animal Resources Commission standards.

DC Generation from Bone Marrow Cultures. The procedure used in these studies was the same as previously described (8, 19). In brief, bone marrow was flushed from the long bones of the limbs and depleted of red cells with ammonium chloride. Bone marrow cells were depleted of lymphocytes, granulocytes, and Ia$^+$ cells using a mixture of mAbs and complement. The mAbs used were 2.43 or 53-6.72 (CD8), GK1.5 (CD4), RA3-3A1/6.1 (CD45R), B21-2 anti-Ia (Tumor Immunology Bank 210, 105, 207, 146, and 229, respectively; American Type Tissue Culture Collection), and RB6-8C5 anti-Gr-1 (provided by DNAX, Palo Alto, CA). Cells were plated in 6-well culture plates (10^6 cells/ml, 3 ml/well) in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 50 mM 2-mercaptoethanol, 10 mM Hepes (pH 7.4), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 3.3 ng/ml GM-CSF (provided by Amgen, Inc., Thousand Oaks, CA). On day 3 of culture, floating cells were gently removed, and fresh medium was added. On day 7 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were collected and replated in 100-mm Petri dishes (10^6 cells/ml, 10 ml/dish). At 10 d of culture, nonadherent cells (DCs) were removed for analysis and immunizations.

The quality of DC preparation was characterized by cell surface marker analysis, morphological analysis, and the ability of the preparation to induce OVA-specific CTLs in immunized mice as previously described (data not shown; references 19, 20).

Vaccination with DCs Pulsed with Tumor Extracts. DCs were washed twice in Opti-MEM medium (GIBCO BRL) and then resuspended at 5–10 × 10^6 cells/ml in 50 ml polypropylene tubes (Falcon, Lincoln Park, NJ). The cationic lipid, DOTAP, (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to deliver tumor extracts into cells. Tumor extracts were obtained by sonicating tumor cells in Opti-MEM (10^7 cells/500 μl) using a Special Ultrasonic Cleaner (Laboratory Supplies Company, Hicksville, NY) and were used without any further manipulation. Tumor extracts (500 μl) and DOTAP (125 μg in 500 μl Opti-MEM medium) were mixed in 12 × 75–mm polystyrene tubes at room temperature for 20 min. The complex was added to the DCs and incubated at 37°C in a water bath with occasional agitation for 25 min. The cells were washed and resuspended in PBS

(10^5 extract-pulsed DCs in 500 μl PBS/mouse) for intraperitoneal immunizations.

Vaccination Using RNA-pulsed DCs. Total RNA was isolated from actively growing tissue culture cells by standard methods as previously described (15). Pulsing DCs with RNA was performed in serum-free Opti-MEM medium (GIBCO BRL) as described for tumor extracts with the following modifications. RNA (25 μg in 250 μl Opti-MEM medium) and DOTAP (50 μg in 250 μl Opti-MEM medium) were mixed in 12 × 75 mm polystyrene tubes at room temperature for 20 min. The complex was added to the DCs (2–5 × 10^6 cells/ml) and incubated at 37°C in a water bath with occasional agitation for 25 min. The cells were washed twice and resuspended in PBS (10^5 RNA-pulsed DCs in 500 μl PBS/mouse) for intraperitoneal immunizations.

PBS, B16 extract from 10^5 cells in PBS, or DCs prepared as described above were injected intraperitoneally in a volume of 500 μl.

Vaccination with GM-CSF–secreting Tumor Cells. Crip cells genetically engineered to produce replication-incompetent recombinant retrovirus with an amphotropic host range and with the ability to encode the cDNA for the murine GM-CSF gene were used as previously described (4, 21). B16 cells were infected by exposure to viral supernatants from these cells in the presence of polybrene (Sigma Chemical Co., St. Louis, MO). GM-CSF production from B16 cells exposed to virus was confirmed by ELISA (Endogen, Cambridge, MA) and standard bioassays (21).

B16 parent cells with the modified GM-CSF gene and the untransfected cells were harvested, washed once in serum-containing medium, and washed twice in PBS. Cell pellets were resuspended in PBS at 10^5/500 μl, irradiated (3,500 centiGray), and injected subcutaneously. The subcutaneous route of administration was chosen because we previously demonstrated that this is the preferred method for administering tumor vaccines (data not shown).

In Vitro Cytotoxicity Assay. In vitro cell–mediated cytotoxicity assays were performed using standard procedures as we previously described (22). In this study, splenocytes obtained from immunized animals and controls were restimulated in vitro for 5 d on monolayers of irradiated and mitomycin C–treated B16 cells or SMA 560 cells. Target cells included SMA 560 and B16 cells.

Implantation of Brain Tumors. B16 cells were harvested by trypsinization, washed twice in Dulbecco's PBS, and mixed with an equal volume of 10% methylcellulose in zinc option medium. The cells (500 cells in a volume of 5 μl) were then implanted into the right caudate nucleus of the brain of C57BL/6 mice by stereotactic injection as previously described (3, 4).

Statistical Analysis. Survival estimates and median survivals were determined using the method of Kaplan and Meier (23). Survival data was compared using Wilcoxon's log-rank test. Student's *t* test was used for calculating the significance of other data. Statistical significance was determined at the <0.05 level.

Results

Vaccination with Bone Marrow-derived DCs Pulsed with Tumor Extract or Tumor RNA Induces Tumor-specific CTLs. To test whether bone marrow–derived DCs pulsed with tumor extracts or tumor RNA are capable of inducing tumor-specific CTLs, we first immunized C57BL/6 mice with three intraperitoneal injections of DCs spaced 1 wk apart. Standard cytotoxicity assays were performed using
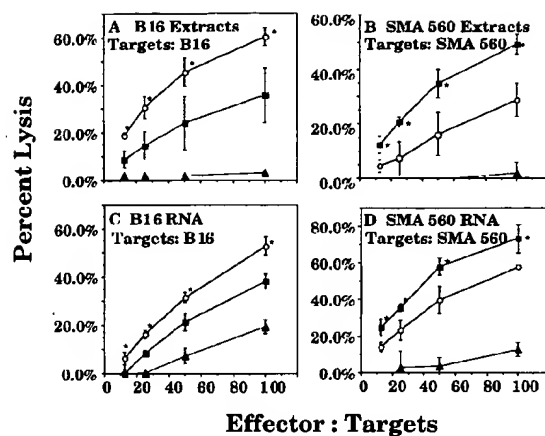
Figure 1. Induction of specific lytic activity against tumor cells by immunization with DC pulsed with tumor extracts or with tumor-derived total RNA. Triplicate C57BL/6 mice were immunized three times with either DC pulsed with (open circles) B16 tumor extract or (filled squares) SMA 560 tumor extract (A and B, respectively), or with DC pulsed with (open circles) B16 tumor RNA or (filled squares) SMA 560 RNA (C and D, respectively) or with PBS (filled triangle). 7 d later, splenocytes were isolated and restimulated for 5 d with irradiated B16 cells (A and C) or SMA 560 cells (B and D). Cytotoxic activity was then measured by chromium release assay using the targets indicated in each panel. *(P <0.05) indicate points of significant differences in lysis when B16-immunized animals are compared to SMA 560–immunized animals. Error bars indicate 1 SD of the mean.

splenocytes harvested from immunized animals 7 d after the third immunization and restimulated for 5 d in vitro with irradiated B16 cells. Cytotoxic activity was tested against B16 cells. We previously demonstrated that the B16 is a poorly immunogenic tumor cell line such that no induction of CTLs occurs after immunization with unmodified parent cells, and such immunization affords no antitumor immunity in vivo (4).

As shown in Fig. 1, immunization using DCs pulsed with either B16 tumor extract or B16 RNA induced B16-specific CTL responses that were statistically significant compared with animals immunized using DCs pulsed with either SMA 560 tumor extract or SMA 560 RNA, or with animals injected using PBS (Fig. 1, A and C). Conversely, splenocytes harvested from groups of animals immunized with SMA 560-pulsed (extract or RNA) DCs specifically lysed SMA 560 targets (Fig. 1, B and D), further demonstrating the tumor-specific nature of these responses. As seen in Fig. 1, high levels of nonspecific lysis are observed when animals are injected with bone marrow–derived DCs pulsed with control antigen, but not those injected with PBS. High levels of nonspecific lysis associated with the use of bone marrow–derived DCs have been previously described and were shown to be dependent upon the presence of sygeneic MHC class II molecules on the immunizing DCs (8) and can be partially overcome by the adherence depletion of antigen-presenting cells before the restimulation of effectors (19).

B16 Tumor Challenge in CNS After Immunization Using DCs Pulsed with Tumor Extracts or RNA. Next, experiments
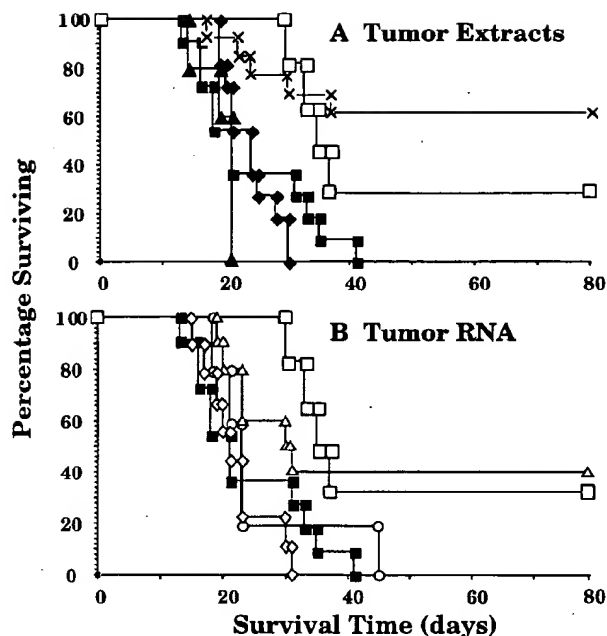

Figure 2. Vaccination with DCs pulsed with tumor extract or tumor RNA protects against CNS challenge with B16 tumor and is equipotent to vaccination with GM-CSF–producing B16 cells. Vaccination of C57BL/6 mice was performed a total of three times before intracranial tumor challenge with B16 cells as described in Materials and Methods. Mice were evaluated daily until death. Data are representative of two experiments performed with similar results. The results are divided in two panels for clarity. The results for the PBS-immunized group (closed squares) and the GM-CSF modified B16 cell immunized group (open squares) are represented in both A and B for ease of comparison. Median days of survival, range, number of animals, and significance compared to PBS-immunized animals based on log-rank analysis for each group are as follows: closed squares, PBS: 21, 13–41, n = 10; X, DC pulsed with B16 tumor extract: >80, 17->80, n = 13, P = 0.0006; closed diamonds, DCs pulsed with SMA 560 extract; 24, 19–30, n = 11, P = 0.50; closed triangles, B16 extract: 21, 14–21, n = 5, P = 0.45; open squares, GM-CSF–modified B16 cells: 36, 30->80, n = 6, P = 0.022; open triangles, DC pulsed with B16 RNA: 31, 19->80, n = 10, P = 0.0001; open diamonds, DC pulsed with SMA 560 RNA: 21, 15–31, n = 9, P = 0.40; open circles, unmodified B16 cells: 23, 18–45, n = 5, P = 0.39.

were performed to determine whether vaccination using B16-pulsed DCs generated specific and protective immunity against B16 tumors within the CNS. Groups of C57BL/6 mice received three intraperitoneal vaccinations spaced 1 wk apart and composed of PBS, B16 extract alone, or DCs pulsed with either B16– or SMA 560–derived extracts or RNA. Mice were then challenged in the brain 1 wk later with 500 viable B16 cells.

As shown in Fig. 2, immunizations with PBS, B16 extract alone, or DCs pulsed with SMA 560 extract or RNA did not protect against CNS challenge with B16 cells. All animals in these groups succumbed to tumor; median survival was between 21 and 24 d (Fig. 2, A and B). In contrast, the median survival of animals undergoing vaccinations of DCs pulsed with B16 extract was significantly prolonged to >80 d (P = 0.0006), with 8 of 13 animals surviving when the experiment was stopped at 80 d (Fig. 2
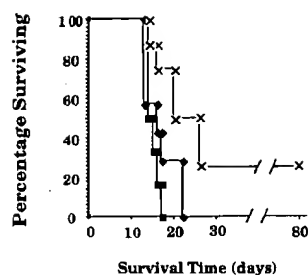
Figure 3. Treatment of established CNS B16 tumors with DCs pulsed with tumor extract prolongs survival. Intracranial tumor challenge with B16 cells was performed first and then, starting 4 d later, C57BL/6 mice were treated with a total of three immunizations as described in Materials and Methods. Mice were evaluated daily until death. Median days of survival, range, number of animals, and significance compared to PBS-immunized animals based on log-rank analysis for each group are as follows: filled squares, PBS: 16, 15–18, $n = 6$; X, DC pulsed with B16 tumor extract: 26, 14–>80, $n = 7$, $P = 0.037$; (filled diamonds), DC pulsed with SMA 560 extract: 16, 15–26, $n = 7$, $P = 0.57$.

A). Likewise, animals receiving vaccinations of DCs pulsed with B16 RNA experienced a significant improvement in median survival to 31 d ($P = 0.0001$), with 4 of 10 animals surviving when the experiment was stopped at 80 d. No statistical significance was demonstrated in the difference between groups immunized using DCs pulsed with either B16 extract or RNA ($P = 0.29$).

It has been convincingly demonstrated that vaccination with tumor cells genetically engineered to secrete various cytokines stimulates a potent immune response against tumors outside the CNS (21, 24, 25). We recently showed that subcutaneous vaccination with B16 cells that are genetically engineered to produce GM-CSF stimulates a potent antitumor immune response against B16 tumors located in the brain and increases the survival of tumor-bearing C57BL/6 mice. It was, therefore, of interest to compare the efficacy of DC-based immunotherapy with immunization using tumor cells having the modified GM-CSF gene. As shown in Fig. 2, A and B, mice vaccinated with GM-CSF gene-modified B16 cells had a median survival of 36 d with two of six mice surviving beyond 80 d, whereas mice vaccinated with B16 cells alone did not exhibit a survival advantage. Vaccination using DCs pulsed with cell extracts (Fig. 2 A) or tumor RNA (Fig. 2 B) was as at least as effective as vaccination using the B16 cells containing modified GM-CSF. Although the median survival of the group vaccinated using DCs pulsed with B16 tumor extract was higher than the group vaccinated with GM-CSF–modified B16 cells, the level of protection achieved with the DC-based vaccines was not statistically greater than that obtained with vaccines using GM-CSF–modified B16 cells ($P = 0.069$).

Prolonged Survival of Mice Bearing CNS B16 Tumors and Treated Using DCs Pulsed with B16 Tumor Extract. In considering the clinical application of a tumor vaccination strategy, it is more realistic to treat animals with tumor present at the time of vaccination. Thus, in the next experiment, 500 B16 cells were implanted in the brain of naive mice, and these mice were treated starting 4 d later, at which point vascularized tumor can be demonstrated histologically (4). Animals received treatments with three intraperitoneal vaccinations spaced 1 wk apart with PBS, DCs pulsed with SMA 560 extract, or DCs pulsed with B16 ex-
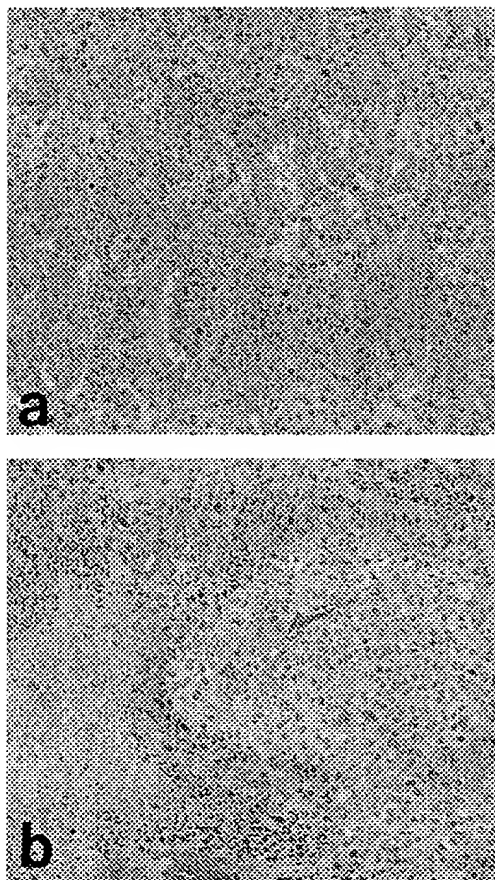
Figure 4. CNS B16 tumors from animals vaccinated with DC pulsed with B16 tumor extract demonstrate large areas of necrosis, hemorrhage, and inflammatory infiltrate (b). No such changes are seen in CNS B16 tumors from animals vaccinated with DC pulsed with control tumor extract (a) or PBS (not shown).

tract (Fig. 3). Mice in the first two groups had median survivals of 16 d. Mice treated with the DCs pulsed with B16 extract had a significantly longer median survival of 26 d ($P = 0.037$), with two of seven animals surviving at 80 d when the experiment was stopped.

Histologic Characterization of CNS Tumors in Immunized Animals. CNS tumors from triplicate animals immunized only once with PBS or DCs pulsed with B16 or control EL-4 tumor extract were examined histologically. Tumors of the group vaccinated with DCs pulsed with B16 extract demonstrated large areas of hemorrhage and necrosis with an associated heavy inflammatory infiltrate composed of both mononuclear cells and polymorphonuclear leukocytes. In comparison, no such areas were observed in either control group (Fig. 4). Outside the immediate peritumoral regions, the brain parenchyma appeared histologically normal.

Discussion

In this study, we showed that immunization with DCs pulsed with either unfractionated tumor extracts or with

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total tumor RNA elicits potent immunity against CNS tumors in mice. We showed that this therapy can be used for both protection against CNS tumor challenge and in the treatment of established tumors. Furthermore, when compared directly, B16-pulsed DCs had potency at least equivalent to GM-CSF–modified B16 vaccines.

A number of barriers exist, in practice, to the treatment of human brain tumors using genetically modified autologous tumor cell vaccines. These vaccines require the considerable tasks of ex vivo purification, culture, expansion, and transfection of tumor specimens, a difficult undertaking even for tumors outside the CNS (6, 7). DC-based vaccines may overcome some of these problems. First, human DCs can be generated from peripheral blood; therefore, supplies are not limited. Secondly, the ex vivo manipulations required to produce antigen-pulsed DCs are simpler than those required for generating autologous tumor cell vaccines.

It has been shown that immunizing nonhuman primates and guinea pigs with human glioblastoma multiforme tissue can induce allergic encephalomyelitis that is lethal (5). Vaccination with unfractionated tumor–derived antigens, such as those possibly contained in an autologous tumor cell vaccine derived from the CNS and modified genetically, may lead to potentially disastrous consequences such as autoimmune encephalitis. This risk may limit the use of such vaccines to a minority of patients: those suffering with brain tumors from which highly purified tumor specimens can not be guaranteed. One approach that may overcome these drawbacks is to use, as a source of antigen, mRNA from tumor cells. In the case of brain tumors, an important advantage is that RNA may allow the use of subtractive hybridization techniques to reduce the concentration of antigens shared between tumor and normal CNS tissue, lessening the potential for autoimmunity.

By demonstrating that vaccines based on DCs pulsed with tumor extracts or RNA are active against CNS tumors and are equipotent to cytokine gene–modified vaccines, these studies establish a basis for future preclinical studies of human DC-based vaccines for treating brain tumors.

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